VOL. XIV, No. 2 APRIL 1961

UNIVERSITY OF HAWAII
LIBRARY
JUL 27 '61

PLANT AND SOIL

International Journal of Plant Nutrition, Plant Chemistry, Soil Microbiology and Soil-borne Plant Diseases

MARTINUS NIJHOFF – PUBLISHER – THE HAGUE

PLANT AND SOIL

International Journal of Plant Nutrition, Plant Chemistry, Soil Microbiology and Soil-borne Plant Diseases

Revue internationale de nutrition des plantes, de chimie végétale, de microbiologie du sol et des maladies des plantes en rapport avec le sol

Internationale Zeitschrift für Pflanzenernährung, Pflanzenchemie, Bodenmikrobiologie und bodenbedingte Pflanzenkrankheiten

Issued under the auspices of the
"Koninklijk Genootschap voor Landbouwwetenschap"
(Royal Netherlands Society of Agricultural Science)

Executive Editors: D. I. Arnon, Berkeley; J. Baeyens, Louvain; F. C. Gerretsen, Groningen; G. W. Harmsen, Groningen; E. G. Mulder, Wageningen; F. J. Richards, London; A. C. Schuffelen, Wageningen; F. Steenbjerg, Copenhagen. **Secretary:** G. H. Arnold, Groningen.

Consulting Editors: Wm. A. Albrecht, Columbia, Mo.; C. Arnaudi, Milan; E. Gäumann, Zurich; L. Gisiger, Liebefeld-Berne; F. G. Gregory, London; A. J. de Groot, Groningen; W. R. C. Handley, Oxford; J. L. Harley, Oxford; J. den Holder, Ghent; V. J. Koningsberger, Utrecht; J. Lavollay, Paris; L. Leyton, Oxford; W. J. Lütjeharms, Bloemfontein; H. Lundegårdh, Uppsala; E. Melin, Uppsala; M. Ødelien, Ås; E. J. Petersen, Copenhagen; C. S. Piper, Adelaide; D. Snow, Reading; H. G. Thornton, Harpenden, Herts.; S. Tovborg Jensen, Copenhagen; A. I. Virtanen, Helsinki; S. A. Waksman, New Brunswick; F. W. Went, Pasadena; Joh^a. Westerdijk, Baarn; L. K. Wiersum, Groningen; J. G. Wood, Adelaide.

The Editors are - if necessary - advised by experts from the Staff of the Institute for Soil Fertility, Groningen

CONTENTS: TABLE DES MATIÈRES: INHALT:

W. J. Rees and *G. H. Sidrak*, Inter-relationship of aluminium and manganese toxicities towards plants. 101

W. L. Lindsay and *J. D. DeMent*, Effectiveness of some iron phosphates as sources of phosphorus for plants 118

S. T. Senewiratne and *D. S. Mikkelsen*, Physiological factors limiting growth and yield of *Oryza Sativa* under unflooded conditions. 127

Y. T. Tchan, L. N. Balaam, R. Hawkes and *F. Draette*, Study of soil algae IV. Estimation of the nutrient status of soil using an algal growth method with special reference to nitrogen and phosphorus 147

E. J. Hewitt, and *G. Bond*, Molybdenum and the fixation of nitrogen in *Casuarina* and *Alnus* root nodules 159

V. V. Rendig and *E. A. McComb*, Effect of nutritional stress on plant composition II. Changes in sugar and amide nitrogen content of normal and sulfur-deficient alfalfa during growth 176

J. B. Weir, The effect of colchicine and indolyl acetic acid on diploid and tetraploid strains of red and white clovers in aseptic and pot culture . . 187

Letter to the editors. *Jens Roll-Hansen*, Some striking observations on the occurrence of fungi in steamed glasshouse soil 197

Printed in the Netherlands
Plant and Soil XIV, 101-198 The Hague April 1961

Plant and Soil XIV, no. 2 April 1961

INTER-RELATIONSHIP OF ALUMINIUM AND MANGANESE TOXICITIES TOWARDS PLANTS

by W. J. REES and G. H. SIDRAK *

Botany Department, University of Birmingham

INTRODUCTION

Investigations into the effect of soil acidity on plant growth have revealed the many other nutritional disturbances accompanying the damaging effect of a high hydrogen-ion content. Among the foremost of these is the so-called induced aluminium and manganese toxicities which are produced in crops sown on acid soils (Hewitt [1]). During an investigation into the problems of plant nutrition on fly-ash (Rees and Sidrak [6]) it was shown that part of the problem was concerned with high concentrations of aluminium which are released to plants under conditions of high pH. Manganese is similarly present in higher than normal soil concentrations, and it has also subsequently been shown that boron exists in toxic concentrations in the fresh ash (Holliday *et al.* [3] and Jones [4]).

This paper contains a detailed account of the effects on growth and mineral status of selected plants when varying levels of aluminium and manganese, together and singly, were applied in water and sand cultures.

MATERIALS AND METHODS

Plants employed

One of the plants used in the investigation was the weed *Atriplex hastata* variety *deltoidea* which is the earliest colonizer of the newly tipped ash. The other two were crop plants, spinach and barley. Spinach was chosen because of its taxonomic connection with *Atriplex*, and barley because it is well known as a sensitive plant to soil abnormalities. Seeds of the crop plants

* Present address: Botany Department, University of Khartoum, Sudan.

were supplied by Messrs. Suttons Ltd., while those of the *Atriplex* used were the hard type of seed as collected from plants found growing on the ash tips. In preparation for the culture experiments the seeds were first germinated for one week in acid-purified sand. They were then transferred to the culture solutions.

Culture solutions

Water-culture solutions were placed in one-litre tall pyrex glass beakers and aerated for nine hours daily. The seedlings were supported in these beakers by cork mats previously impregnated with wax. Each mat supported three seedlings. Eight-and-a-half-inch clay pots previously painted inside with three coats of pure bitumen solution were employed for the sand-culture work. Each pot contained 7 kg of sand. The culture solution used had element concentrations similar to that of Hewitt [2] and a pH of 5·5. Water-culture solutions were replaced every two weeks while sand cultures received 500 ml culture solution daily. Sand was previously purified with acid and distilled water.

Sampling

Fresh and dry weights of individual plant tops were recorded at harvest. Plant roots could not be treated in this way. Roots from each container were pooled and a mean weight recorded. The oven-dried tops and roots from each container were ground to powder with a glass pestle and mortar in preparation for chemical analysis.

Chemical analysis

The wet-digestion and analytical methods described in Rees and Sidrak [6] were used.

EXPERIMENTAL RESULTS

a. EXPERIMENTS WITH ALUMINIUM

In November, 1954 one-week old seedlings of *Atriplex*, spinach, and barley were transferred to full culture solution for 7 days. They were then given six treatments containing 0, 10, 20, 30, 40, 50, and 60 ppm Al. Aluminium was supplied in two forms, as sulphate and EDTA complex and, in the case of barley and spinach, the EDTA compound was applied in sand culture as it proved fatal to both plants when given in water culture. When aluminium was applied as sulphate, phosphate precipitation was avoided by adopting the changing technique developed by McLean and Gilbert [5] *i.e.* seedlings were grown alternately for 2 days in culture with Al but without phosphate and then in normal full culture. Controls were treated similarly but without additional Al. An appropriate amount of sodium sulphate was added to the controls in the series to allow

for the sodium content of the EDTA aluminium supplied by "The Geigy Company Ltd".

Symptoms induced

i. Aluminium sulphate series. *Atriplex*, although somewhat smaller than the controls, showed no visual symptoms, Plate 1A. On the other hand the spinach plants were affected quite early by extra Al, leaves developing diffuse chlorosis and most plants flowering prematurely, Plate 1C. Barley grew normally in all treatments for the first three weeks, but later developed tip die-back on the younger leaves while the more mature leaves became yellow and brittle, Plate 1B. The roots of these plants were severely affected by aluminium, they became less turgid than is normal, developing dwarfed laterals with spatulate tips, Fig. 1, and the growing points showed signs of breakdown of the tissue.

ii. Aluminium EDTA series. The growth of *Atriplex* in all concentrations of Al-EDTA exceeded that of the control, Plate 2. In contrast spinach and barley were seriously affected in a manner somewhat similar to i. above.

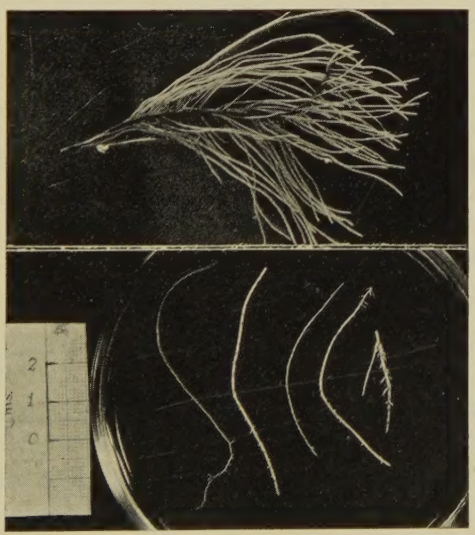


Fig. 1. The general appearance of lateral roots of barley grown in a culture with and without $Al_2(SO_4)_3$.
A: control, B: 20 ppm Al.

Yield data

When the plants were nine weeks old the experiment was terminated and individual plants weighed and dried for chemical analyses. The results of these measurements appear in Fig. 2.

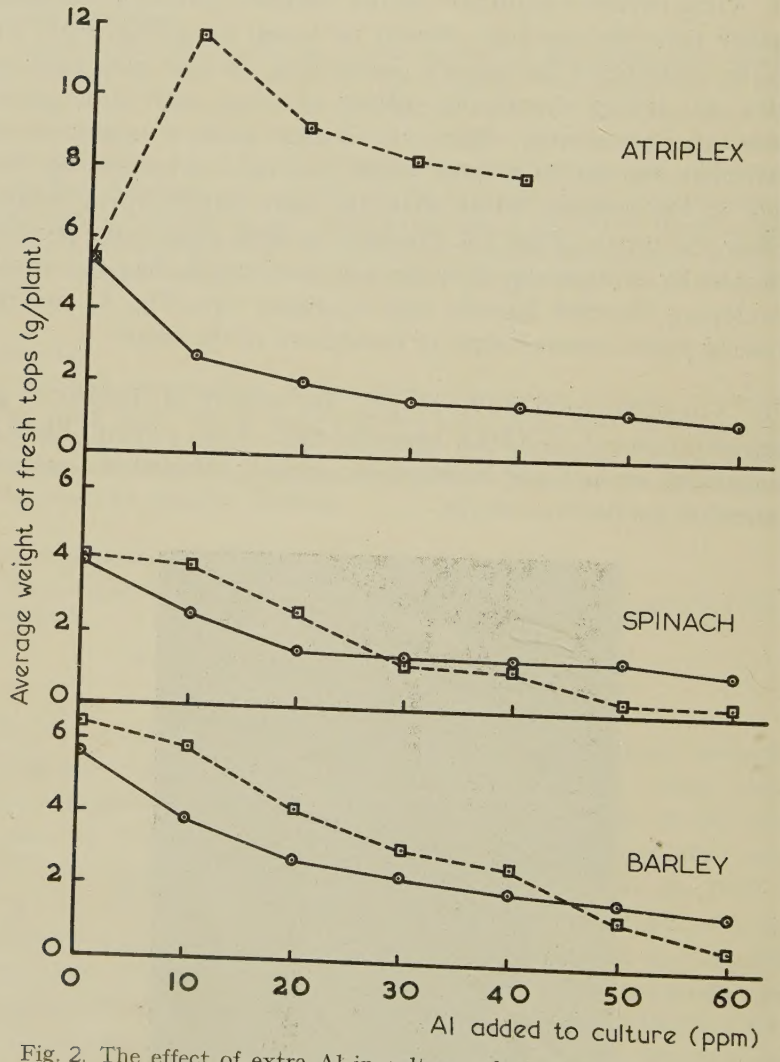


Fig. 2. The effect of extra Al in culture solution on plant yields.
——— $Al_2(SO_4)_3$
– – – Al-EDTA.

Mineral status

The dry plant material (tops in the case of barley and leaves for the remainder) was analysed for six major nutrients, together with Al and Mn (Table 1). Several interesting points emerge from these analyses. The nitrogen content of barley is significantly reduced by quite low concentrations of both Al compounds. In contrast, higher concentrations of Al are required to bring about similar effects on nitrogen uptake in the case of spinach.

The nitrogen status of *Atriplex* is unaffected by $Al_2(SO_4)_3$, whilst the EDTA compound increases quite materially nitrogen absorption. The phosphate content of *Atriplex* also behaves somewhat similarly. Al-EDTA application increases spinach and barley phosphate also. The phosphate status of the latter plant however is slightly lowered by the small concentration of 10 ppm when aluminium is applied as $Al_2(SO_4)_3$. While addition of Al-EDTA increases uptake of K in barley, in other instances potash content is not significantly affected. On the other hand the Ca-status of both crops is very significantly lowered by Al-application. In the case of *Atriplex*, however, it requires a concentration of some 60 ppm aluminium sulphate to reduce the Ca-uptake significantly.

The Mg-status of the three plants follows the Ca-trends quite closely. Application of $Al_2(SO_4)_3$ does not affect the Fe-status very considerably, but increased concentrations of the EDTA induce very high Fe-uptake by both barley and spinach. In all cases plants accumulate more Al as the concentration of this metal is increased in the culture. The Mn status of spinach is likewise increased in this series. In all other cases Mn is reduced. Indeed in barley EDTA series, this reduction is to less than one-third.

b. EXPERIMENTS WITH MANGANESE

Experiments in this series were commenced during April, 1955, when seedlings of the three plants treated as under *a* above were divided into two groups, one receiving Mn as sulphate, the other Mn as the EDTA complex. Four levels of Mn were employed, 5, 25, 50, and 100 ppm respectively, in duplicate for each group.

Symptoms induced

The effect of $MnSO_4$ on *Atriplex* was very harmful in concentrations above 5 ppm. At an early stage the apex of the lamina

TABLE 1

Mineral status of plants given various levels of aluminium
(Mean of three determinations – dry wt. bases)

Al added ppm	Per cent					Ppm		
	N	PO_4	K_2O	CaO	Mg	Fe	Mn	Al
Atriplex								
$Al_2(SO_4)_3$								
0	4·72	1·90	4·75	1·43	0·79	264	117	79
10	4·62	1·80	4·75	1·34	0·75	253	117	237
20	4·62	1·88	4·32	1·42	0·74	238	122	479
30	4·86	1·96	4·30	1·43	0·73	250	114	562
40	4·83	1·80	4·30	1·32	0·72	264	94	594
50	4·62	1·84	4·26	1·32	0·71	245	85	669
60	4·70	1·82	4·25	1·25	0·69	250	57	743
LSD $P = 0.05$	0·11	0·13	0·89	0·13	0·05	13	3	16
Al-EDTA								
0	4·72	1·90	4·75	1·43	0·79	264	117	79
10	5·10	2·40	4·64	1·31	0·89	265	86	136
20	5·15	2·70	4·82	1·34	0·89	263	72	248
30	5·20	3·20	4·72	1·25	0·93	282	54	380
40	5·10	3·40	4.60	1·31	0·98	286	34	490
LSD $P = 0.05$	0·11	0·16	1·04	0·14	0·04	14	4	12
Spinach								
$Al_2(SO_4)_3$								
0	4·96	3·08	8·96	1·25	0·84	279	70	64
10	5·02	3·08	8·90	0·98	0·71	283	40	83
20	4·96	3·00	8·72	0·87	0·64	276	40	98
30	4·80	2·96	8·60	0·87	0·62	280	40	106
40	4·24	2·96	8·50	0·87	0·60	281	40	124
LSD $P = 0.05$	0·19	0·17	1·45	0·14	0·12	9	3	8
Al-EDTA (sand culture)								
0	5·60	2·60	7·25	2·04	0·84	207	63	103
10	5·60	2·70	7·22	1·48	0·82	204	73	144
20	5·70	2·78	7·20	1·48	0·83	314	72	192
30	5·50	2·81	7·10	1·39	0·83	359	82	244
40	5·30	2·92	7·25	1·35	0·68	358	82	311
50	5·20	3·13	6·95	1·26	0·64	442	80	355
60	5·30	3·23	6·90	1·15	0·60	496	91	488
LSD $P = 0.05$	0·19	0·09	1·02	0·39	0·05	13	10	23
Barley								
$Al_2(SO_4)_3$								
0	5·12	1·72	7·52	1·12	0·31	216	38	38
10	4·84	1·45	7·60	0·96	0·31	238	25	51
20	4·88	1·42	7·52	0·96	0·32	250	25	62
30	4·72	1·42	7·68	0·92	0·32	253	25	68
40	4·84	1·44	7·52	0·86	0·31	253	24	78
50	4·78	1·50	7·52	0·75	0·30	253	24	108
60	4·86	1·49	7·52	0·75	0·29	255	24	125
LSD $P = 0.05$	0·13	0·19	0·88	0·16	0·05	15	4	6
Al-EDTA (sand culture)								
0	4·70	1·07	6·00	1·16	0·27	213	34	39
10	4·50	1·12	6·06	0·90	0·17	292	19	38
20	4·40	1·27	6·86	0·86	0·16	350	19	36
30	4·45	1·35	6·95	0·75	0·16	363	17	35
40	4·45	1·56	7·20	0·73	0·16	360	9	44
50	4·20	1·99	7·80	0·68	0·15	383	9	66
60	4·10	2·01	7·80	0·65	0·15	403	9	66
LSD $P = 0.05$	0·22	0·14	0·79	0·15	0·05	13	3	5

became yellowish brown, which later spread to the entire leaf blade. EDTA-treated plants, although dwarfed at higher concentrations of Mn, did not show visual symptoms. In contrast, both spinach and barley developed leaf chlorosis and yellowing with both forms of manganese. Barley leaves showed minute, brown spots in the lamina.

Yield data

The experiment was ended when the plants were eight weeks old. Fig. 3 gives the correlation between the fresh weight of the plants and the extra amount of manganese added to the culture solution. Dry weights of plants follow the fresh weights.

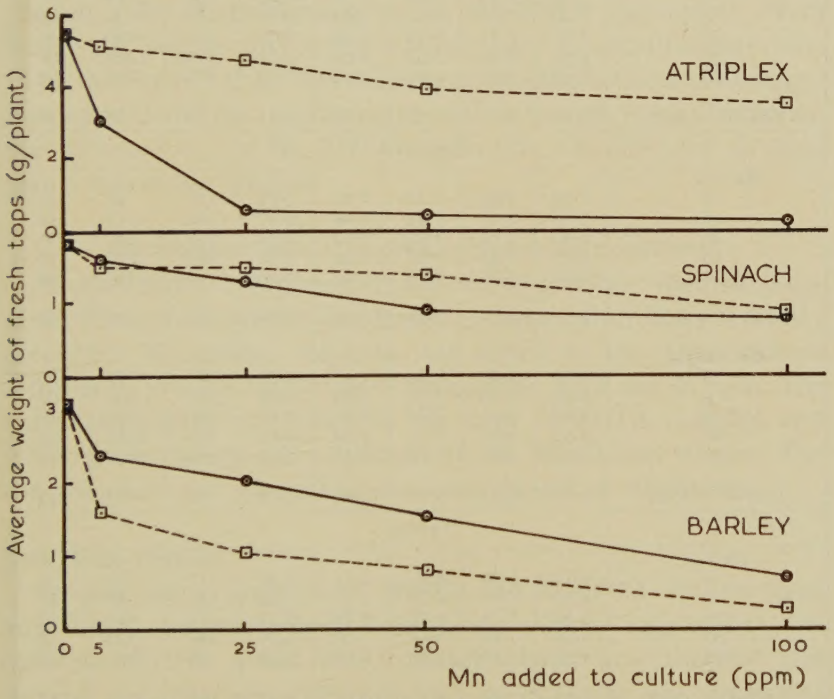


Fig. 3. The effect of extra Mn in culture solution on plant yields.
——— $MnSO_4$
– – – Mn-EDTA.

Mineral status

Results of chemical analyses of dry plant materials are given in Table 2. The extra manganese added to the culture medium in the form of sulphate did not affect the nitrogen, phosphorus, potassium

TABLE 2

Mineral status of plants given various levels of manganese
(Mean of three determinations – dry wt. bases)

Mn added ppm	Per cent					Ppm		
	N	PO_4	K_2O	CaO	Mg	Fe	Mn	Al
Atriplex								
$MnSO_4$								
0	5·70	2·00	5·70	1·37	0·83	232	171	66
5	5·80	1·98	5·68	1·92	0·95	215	520	70
25	5·75	2·08	5·63	2·10	1·60	147	1280	71
50	5·70	2·02	5·71	2·95	1·73	113	1800	70
LSD $P = 0·05$	0·94	0·09	2·12	0·92	0·14	29	30	12
Mn-EDTA								
0	5·70	2·00	5·70	1·37	0·83	232	171	66
5	5·20	2·00	5·60	2·04	0·89	230	700	60
25	4·95	1·97	5·23	2·02	1·00	411	1180	88
50	4·60	2·30	5·62	2·04	1·02	490	1280	106
100	4·60	2·53	4·26	2·12	1·13	592	1700	126
LSD $P = 0·05$	0·76	0·07	1·57	0·79	0·11	30	51	15
Spinach								
$MnSO_4$								
0	5·50	2·26	7·20	1·45	0·79	196	95	88
5	5·55	2·16	7·25	1·40	0·70	182	117	90
25	5·40	2·15	7·20	1·37	0·76	162	330	96
50	5·45	2·25	7·20	1·32	0·83	146	720	94
100	5·55	2·23	7·15	1·21	0·90	110	1320	96
LSD $P = 0·05$	0·91	0·09	2·31	0·86	0·08	18	43	14
Mn-EDTA								
0	5·50	2·26	7·20	1·45	0·79	196	95	88
5	5·40	2·30	7·05	2·04	0·76	235	200	106
25	4·80	2·67	7·30	2·20	0·87	295	720	116
50	4·60	3·12	7·60	2·34	0·92	405	1060	122
100	4·30	3·35	7·62	2·33	1·02	600	1480	132
LSD $P = 0·05$	0·91	0·15	2·14	0·91	0·09	28	47	23
Barley								
$MnSO_4$								
0	4·70	1·20	6·70	1·31	0·26	117	38	36
5	4·35	1·15	6·70	1·16	0·24	116	93	37
25	4·30	1·15	6·70	1·08	0·29	112	453	37
50	4·25	1·18	6·68	1·00	0·29	106	720	42
100	4·20	1·15	6·50	0·86	0·29	99	920	48
LSD $P = 0·05$	1·17	0·06	1·83	0·54	0·04	14	34	6
Mn-EDTA								
0	4·70	1·20	6·70	1·31	0·26	117	38	36
5	4·65	1·35	7·00	1·33	0·26	138	110	42
25	4·40	1·60	7·00	1·61	0·28	180	360	58
50	4·40	1·85	7·20	1·65	0·28	215	700	60
100	4·20	2·20	7·32	1·80	0·30	263	1573	62
LSD $P = 0·05$	1·15	0·06	2·05	1·08	0·04	15	41	10

or aluminium status of the three plants. It had also no effect on the calcium and magnesium contents of both spinach and barley, but it caused an increase of these two elements in *Atriplex* leaves. In all three plants the application of manganese sulphate suppressed the iron content of their tops and increased their manganese content.

When the extra manganese was supplied to the culture medium as Mn-EDTA, it did not affect materially the nitrogen nor the potassium of the three plants, but caused an increase in their phosphorus and iron status. The plant calcium showed a tendency to increase, but not significantly. *Atriplex* and spinach magnesium showed a direct relationship to the Mn-EDTA application. With barley the magnesium content was increased to a small extent only. In all three plants there was a direct relationship between the manganese applied and that accumulated in their tissues. Plant aluminium was increased by the Mn-EDTA application; the effect for all three plants was rather similar.

c. EXPERIMENTS WITH ALUMINIUM AND MANGANESE COMBINED

Another series of experiments was set up in 1955, when seedlings of the three plants were transplanted to water cultures as previously described. Manganese sulphate was added to the basic culture solution to provide two levels of manganese, *viz* 5 and 25 ppm. The extra aluminium was added in the form of EDTA complex and varied in the two groups to provide 10, 20, 30, 40, and 50 ppm. The control plants did not receive extra manganese or aluminium.

Symptoms induced

Atriplex plants given extra $MnSO_4$ and Al-EDTA did not show any visual symptoms apart from being slightly bigger than the controls. On the other hand, spinach plants were dwarfed and showed leaf chlorosis, yellowing and tip die-back; the symptoms were aggravated by increasing the aluminium concentration. Barley plants acquired similar symptoms to spinach with the addition of brown specks on the leaf blades.

Yield data

Fig. 4 shows the relationship between metal application and plant yields as compared with the controls.

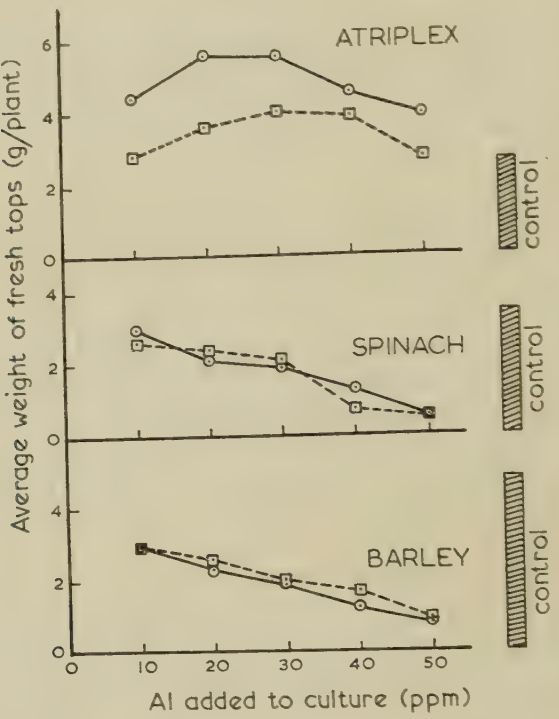


Fig. 4. The effect of Al-EDTA in culture solution on plant yields at two levels of Mn.
——— 5 ppm Mn
– – – 25 ppm Mn.

Mineral status

Chemical analyses of the plant tops (Table 3) reveal that the application of manganese and aluminium to the culture solution had no effect on the nitrogen content of the three plants, while their phosphorus and aluminium contents showed a direct relationship to the amount of Al-EDTA applied at both levels of manganese. The concentration of other elements in plant tissues varied between the various species. In *Atriplex* and spinach potassium and magnesium were not affected by the treatments, while in barley potassium was increased when the aluminium exceeded 30 ppm and magnesium was suppressed by increasing aluminium application at both manganese levels. Increasing the manganese application caused an increase in the calcium content of *Atriplex* but not that

TABLE 3

Mineral status of plants in water cultures with varying aluminium concentrations at two manganese levels (Dry weight bases)

Metal added ppm		Per cent					Ppm		
Mn	Al	N	PO_4	K_2O	CaO	Mg	Fe	Mn	Al
Atriplex									
0	0	5·15	2·00	5·10	1·45	0·78	226	115	82
5	10	5·20	2·15	5·05	1·40	0·75	229	275	116
5	20	4·94	2·20	4·90	1·43	0·75	231	195	175
5	30	5·15	2·40	4·86	1·43	0·75	230	150	215
5	40	4·95	2·45	4·82	1·43	0·75	237	126	405
5	50	5·15	2·50	4·70	1·28	0·75	238	100	555
25	10	5·20	2·25	5·10	1·83	0·82	230	1162	111
25	20	5·30	2·50	5·05	1·80	0·84	232	1106	181
25	30	5·25	2·51	4·98	1·88	0·83	242	775	208
25	40	5·20	2·55	4·96	1·88	0·85	237	725	387
25	50	5·20	2·70	4·92	1·88	0·84	235	512	475
LSD $P = 0·05$		0·17	0·05	0·79	0·16	0·05	13	25	11
Spinach									
0	0	5·60	2·36	7·16	1·48	0·81	210	66	98
5	10	5·55	2·61	7·14	1·40	0·81	218	124	144
5	20	5·46	2·90	7·05	1·39	0·81	218	142	148
5	30	5·46	3·10	6·92	1·34	0·81	221	146	269
5	40	5·45	3·26	6·88	1·30	0·82	261	152	314
5	50	5·45	3·32	6·83	1·25	0·82	279	150	324
25	10	5·50	2·50	7·17	1·40	0·83	147	402	136
25	20	5·50	2·75	6·96	1·35	0·83	130	580	153
25	30	5·50	3·20	6·89	1·30	0·84	130	580	286
25	40	5·60	3·28	6·83	1·30	0·85	130	490	300
25	50	5·55	3·40	6·78	1·25	0·85	117	400	332
LSD $P = 0·05$		0·16	0·05	0·81	0·15	0·05	11	10	7
Barley									
0	0	4·43	1·26	6·35	1·20	0·26	137	40	34
5	10	4·60	1·82	6·52	1·05	0·22	137	78	46
5	20	4·60	2·30	6·56	0·93	0·20	147	50	55
5	30	4·60	2·67	7·05	0·88	0·20	208	50	61
5	40	4·30	3·12	7·20	0·78	0·19	208	50	68
5	50	4·26	3·36	7·41	0·75	0·16	213	50	102
25	10	4·30	1·80	6·80	0·95	0·20	133	290	51
25	20	4·30	2·38	6·90	0·84	0·18	135	260	55
25	30	4·35	2·76	7·20	0·75	0·17	167	225	55
25	40	4·30	3·15	7·40	0·75	0·17	188	210	61
25	50	4·50	3·42	7·50	0·65	0·17	208	180	111
LSD $P = 0·05$		0·16	0·07	0·75	0·10	0·04	11	6	5

of spinach and barley, while the Al-EDTA did not affect the *Atriplex*, calcium caused an increase in the calcium status of spinach leaves and a decrease in that of barley tops. The manganese content of both *Atriplex* and barley was increased by the manganese application but suppressed by the aluminium, while in the case of spinach the Al-EDTA caused a slight fluctuation in the manganese content of the leaves. The iron status of *Atriplex* was not affected by the treatments, but in spinach it was greatly reduced at the higher manganese level. In barley, however, the iron content of the tops was gradually increased by increasing the Al-EDTA at both levels of manganese.

DISCUSSION

The effects of aluminium and manganese on plant growth as shown in the preceding paragraphs may be considered from four aspects; the action of the two metals Al and Mn singly and in conjunction, along with results obtained when these metals are used as the EDTA complex.

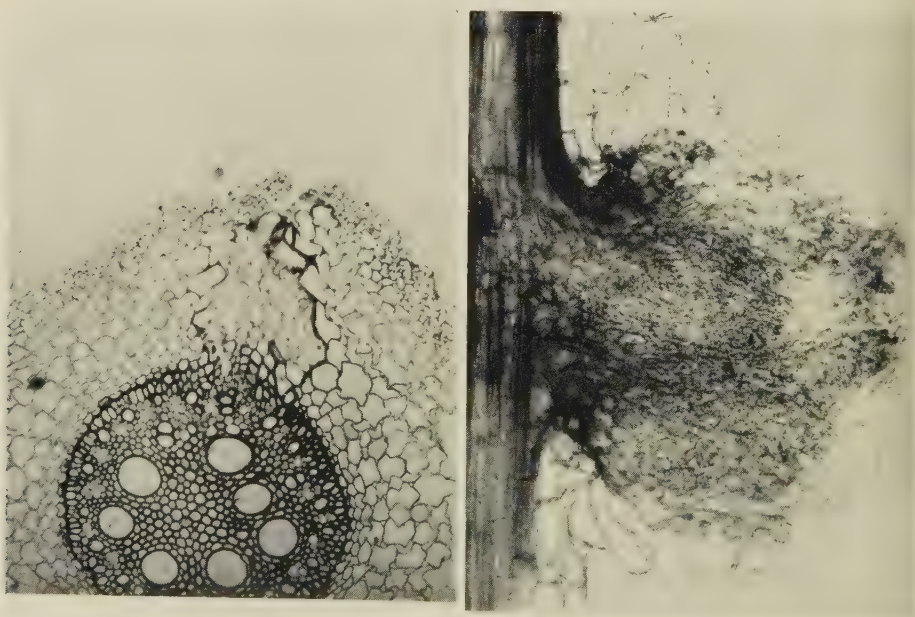
1. *Effect of aluminium*

It is well known that analysis of Al-toxicity effects is often complicated by induced phosphorus-deficiency symptoms. As already mentioned on p. 102 precautions were taken to overcome this difficulty by application of Al and phosphate to the plant independently, and the results obtained are therefore regarded as effects of true Al-toxicity.

Higher concentrations of this metal as sulphate decrease both fresh- and dry-weight yields, but visual symptoms due to Al appeared only on spinach and barley. These symptoms undoubtedly are due to the Al-ion itself, since plant phosphorus status was normal in the sulphate series and above the control in the EDTA series; the latter also produced symptoms similar to the sulphate series. The leaf yellowing cannot be attributed to Fe-deficiency since its level was quite normal, contrary to the findings of Rorison [7] for *Onobrychis sativa*. It is, however, within the range of possibility that Al inactivates the Fe within the cell. High Al, however, does significantly lower leaf Mn in the three plants. No doubt disturbances in the leaves of spinach and barley could well be caused in part by the Al-effect on roots. Our former findings [8]

Plate 1. *Atriplex* (**A**), spinach (**B**), and barley (**C**) plants grown at first in a water culture solution and then transferred to another containing extra $Al_2(SO_4)_3$. *From left to right:* 0 (control), 10, 20, 30, 40, 50, and 60 ppm Al extra.

Plate 2. *Atriplex* plants grown at different levels of Al-EDTA. *From left to right:* 0 (control), 10, 20, 30, and 40 ppm Al extra.



A **B**

Plate 3. Development of lateral roots in barley plants produced at 20 ppm Al as sulphate. **A**, part of cross section; **B**, part of longitudinal section.

are in agreement with those of Rorison [7] that high Al deforms lateral roots. The detailed cytology of these malformations still requires elucidation. However, sections taken from barley roots produced at 20 ppm Al as sulphate during our 1954 experiments show that the lateral root either fails to penetrate the cortex (Plate 3A) or after penetration it forms an irregular mass of parenchyma cells in the form of a small tumour (Plate 3B).

The role of Al in the physiology of plants still remains obscure. Quite a variety of physiological effects have been attributed to it, and of these one which it would appear appropriate to mention here is its effect on cell organization, *e.g.* that it may harden the protoplasm (Szücs [11]) and combine with the colloidal salts, rendering the cell wall impermeable (Stoklasa [9 10]). This effect of Al is by no means confined to the roots. Indeed Al-treated plants accumulate large amounts of this element in their foliage, which in barley produces stiffening of the laminae and so causes an upright habit. In order to explore this possibility further, in 1955 barley leaf tips were injected with varying concentrations of Al and the results compared with others from control leaves injected with distilled

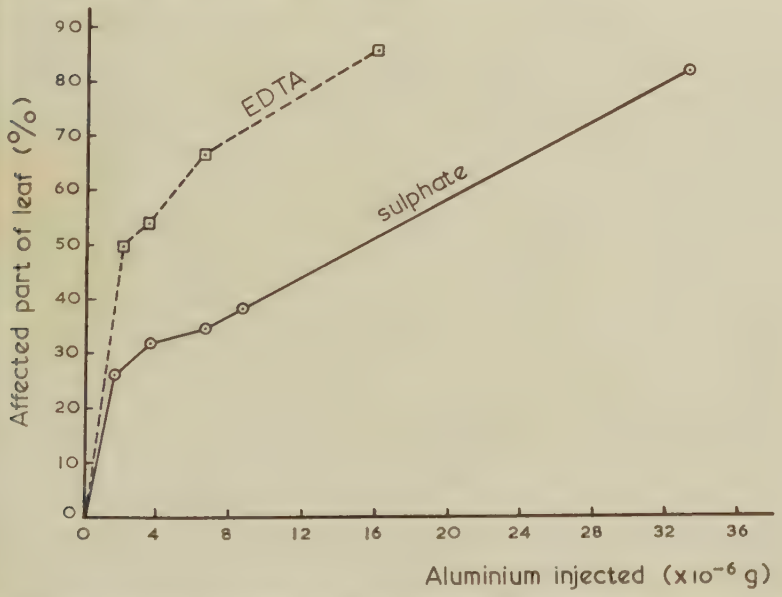


Fig. 5. The relationship between concentration of Al injected into barley leaves and the amount of leaf necrosis.

water. Results of these treatments showed that the degree of yellowing and stiffening of leaf lamina is clearly related to the concentration of Al injected. It is also interesting to notice that Al-EDTA is more effective in producing these changes than the corresponding concentration of $Al_2(SO_4)_3$ (Fig. 5).

Another important effect of Al on spinach and barley is a reduction in leaf Ca-content and consequent disturbance of the K/Ca balance. Table 4 shows that there is a close correlation between the symptoms produced and the rise in K/Ca ratio.

TABLE 4

Effect of Al-treatment on the yield, K/Ca ratio and symptoms

Plant species	Al added ppm (as sulphate)	Yield/plant g	K/Ca	Symptoms
Atriplex	0	5·40	2·0	none
	10	2·70	2·1	none
	20	2·04	1·8	none
	30	1·55	1·8	none
	40	1·49	1·9	none
	50	1·27	1·9	none
	60	1·05	2·0	none
Spinach	0	3·94	4·3	none
	10	2·51	5·4	none
	20	1·53	6·0	slight
	30	1·45	6·1	moderate
	40	1·38	5·8	severe
Barley	0	5·63	4·0	none
	10	3·85	4·7	slight
	20	2·74	4·7	slight
	30	2·31	5·0	moderate
	40	2·00	5·3	moderate
	50	1·78	6·0	severe
	60	1·50	6·0	severe

2. *Effect of manganese*

Here again there is a loss of fresh- and dry-weight yields accompanied by visual symptoms as Mn-application is increased. *Atriplex* is particularly sensitive in this respect, although it shows no symptoms under Al-treatments. A possible explanation of this disturbance may be that it is related to the high accumulation of Mn and consequent reduction of the Fe/Mn ratio, as seen in Table 5. Mn upsets the K/Ca ratio also, reducing its value to less than one

half normal in the case of *Atriplex*. It will be remembered that Al affects the K/Ca ratio of spinach and barley, but not that of *Atriplex*.

TABLE 5

Effect of Mn-treatment on yield, Fe/Mn ratio and symptoms

	Mn added ppm (as sulphate)	Yield/plant g	Fe/Mn	Symptoms
Atriplex	0	5·44	1·36	none
	5	3·06	0·41	none
	25	0·60	0·11	medium
	50	0·47	0·06	severe
Spinach	0	1·82	2·06	none
	5	1·62	1·56	medium
	25	1·32	0·49	medium
	50	0·93	0·20	severe
	100	0·85	0·08	severe
Barley	0	3·14	3·08	none
	5	2·42	1·25	medium
	25	2·04	0·25	severe
	50	1·58	0·15	severe
	100	0·73	0·11	severe

3. *Effect of EDTA complex*

This complex has a quite different effect on each of the three plants whether presented as Al or Mn. Considering first Al as EDTA, *Atriplex* benefits quite markedly from this substance (see Plate 2). Yields obtained greatly exceed control (see Fig. 2). Barley and spinach, on the other hand, had their yields depressed. Indeed, in some cases Al-EDTA was more serious in its effect than the corresponding sulphate application, while symptoms were likewise more pronounced. Neither plant survived Al-EDTA in water culture. A possible explanation of the difference in behaviour of *Atriplex* may lie in the effect of this complex on the Mg-content of the three plants. In *Atriplex* this element increases, but it declines in the other two plants. Al-EDTA also reduces the Ca-status of both spinach and barley but it does not affect that of *Atriplex*. The superiority of the Al-EDTA *Atriplex* as compared with its control and Al-EDTA spinach and barley, may be related therefore to an enhanced Mg-status and a failure of Al-EDTA to lower the Ca-content.

Mn-EDTA was much less drastic than sulphate towards *Atriplex* and spinach, but barley was very susceptible to it. Mn-EDTA also

increased Mg in *Atriplex* and spinach, but did not affect barley Mg. However, it is very interesting to note that the EDTA complex markedly enhanced both phosphorus and iron in the three plants. The reason for this increase is at the moment very speculative, but it would be interesting to ascertain if the phenomenon is common to all plants.

4. *Effect of Al and Mn in conjunction*

Finally it may be said that the effect of Al and Mn in conjunction on fresh and dry weights as well as on mineral status of the three plants is a combination of the action of the individual elements. It is notable, however, that Al alone and Al + Mn did not produce visual symptoms in *Atriplex* whereas Mn alone caused severe dwarfing and leaf burning. Reference to the tables shows that Al protects the plant from excessive Mn and the harmful effects resulting from this excess. This may have an interesting ecological significance for plants which naturally grow on soils under conditions of high concentrations of both available Al and Mn, where the normal growth appears quite unaffected by this soil condition as in the case shown to be true for *Atriplex*. A soil condition of excessive Mn- and normal Al-concentrations might seriously affect or even prevent the survival of such plants. On the other hand, the growth of crop plants, spinach and barley, in high Mn-concentrations are not protected by the addition of extra Al and it is a well known fact that most crops will not grow satisfactorily under similar conditions in the field unless the high available Al and Mn is lowered by addition of lime. Conversely, the so-called calcifuge plants appear, like *Atriplex*, to have acquired a physiological immunity to high Al and Mn when present together. Undoubtedly there is a need for an elaboration of the experiments on the lines of those described in this paper to include more of the so-called calcifuges in order to ascertain if their physiology in response to Al and Mn is similar to that of *Atriplex*.

SUMMARY

1. *Atriplex hastata* var. *deltoidea*, spinach and barley were grown in water and sand cultures to which extra aluminium and manganese, singly and in combination, were added in increasing amounts.

2. The precipitation of phosphates by Al was avoided by developing the changing technique and by using the Al-EDTA complex.

3. $Al_2(SO_4)_3$ caused a decrease in the yield of the three plants and produced chlorosis and tip die-back in spinach and barley, but not in *Atriplex*. It also affected the growth of barley roots.

4. *Atriplex* was seriously depressed by $MnSO_4$ treatments and its leaves became brown and dry at concentrations above 5 ppm Mn. Spinach and barley plants were also affected by $MnSO_4$ and the older leaves were chlorotic. In addition, barley leaves developed dark-brown spots.

5. The use of EDTA complex was beneficial to *Atriplex* in all cases but harmful to both spinach and barley.

6. In all cases EDTA complex markedly enhanced P- and Fe-absorption.

7. The toxic effects of Al were correlated with its effect on the K/Ca balance of the plants and its possible effects on the protoplasm and cell walls.

8. The destructive effect of Mn was attributed mainly to the change in the Fe/Mn ratio and in part to the disturbed K/Ca ratio.

9. The difference in behaviour of *Atriplex* on the one hand, and spinach and barley on the other towards EDTA is thought to depend on the increase in *Atriplex* Mg, and the decrease in spinach and barley Mg caused by this compound.

10. It is suggested that Al protects *Atriplex* from the harmful effects of Mn.

Received August 13, 1959

LITERATURE CITED

1 Hewitt, E. J., A biological approach to the problems of soil acidity. Intern. Soc. Soil Sci. Trans. Dublin 1952, **1**, 107 (1953).
2 Hewitt, E. J., Sand and water culture methods used in the study of plant nutrition. Commonwealth Agr. Tech. Bur. Commun. No. **22** (1952).
3 Holliday, R., Hodgson, D. R., and Townsend, W. N., Plant growth on 'Fly-Ash'. Nature (London) **181**, 1079 (1958).
4 Jones, L. H., The nature of power station ash and its effect on plant growth. Ph. D. Thesis, Birmingham University (1958).
5 McLean, F. T. and Gilbert, B. E., The relative aluminium tolerance of crop plants. Soil Sci. **24**, 163 (1927).
6 Rees, W. J. and Sidrak, G. H., Plant nutrition on 'Fly-ash'. Plant and Soil **8**, 141 (1956).
7 Rorison, I. H., The effect of aluminium on legume nutrition. *In:* Nutrition of Legumes. Proc. Univ. Nottingham **43** (1958).
8 Sidrak, G. H., Plant nutrition on power station ash. Ph. D. Thesis, Birmingham University (1955).
9 Stoklasa, J., Catalytic fertilizers for sugar beets. Bl. Zuckerrüb. **18**, 193 (1911).
10 Stoklasa, J. *et al.*, Über die Resorption des Aluminiumions durch das Wurzelsystem der Pflanzen. Biochem. Z. **128**, 35 (1922).
11 Szücz, J., Über einige charakteristische Wirkungen des Aluminiumions auf das Protoplasma. Jahrb. Wiss. Botan. **52**, 269 (1913).

Plant and Soil XIV, no. 2 April 1961

EFFECTIVENESS OF SOME IRON PHOSPHATES AS SOURCES OF PHOSPHORUS FOR PLANTS

by W. L. LINDSAY and J. D. DeMENT

Soils and Fertilizer Research Branch, Division of Agricultural Relations, Tennessee Valley Authority, Wilson Dam, Alabama

INTRODUCTION

Iron phosphates are found among the reaction products of phosphate fertilizers in soils [10 14]. With fertilizers such as monocalcium phosphate, the acidic solution moving into the soil dissolves considerable quantities of iron, which, in turn, precipitates part of the fertilizer phosphorus [14]. Fractionization studies of soil phosphorus [4 12] also indicate that a considerable portion of the phosphorus in soils is frequently present as iron-bound phosphate. The question arises whether the iron phosphates formed in soil are available to plants.

Previous studies [1 7 16 18 20] have shown differences in the phosphorus availability of certain natural and synthetic iron phosphates. Fujiwara [7 8 9] pointed out that acidic iron phosphates are generally more available than the more basic precipitates. Iron phosphates generally gave better yields of paddy rice grown under reducing conditions than for well-aerated crops on upland soils.

A meaningful evaluation of the availability of iron-containing reaction products from fertilizers requires (1) a knowledge of the types of compounds likely to occur in soils, and (2) the preparation and testing of pure compounds which have been sufficiently characterized in order to identify the chemical phases present. In this regard, much of the work reported in the literature on the availability of iron phosphates is of limited value. In most cases little attention has been given whether a material was amorphous or crystalline, or whether it consisted of a single phase or a mixture of several phosphate compounds.

The iron phosphate compounds selected for the present study are representative of those which may precipitate under conditions that occur in soils. The materials tested were first examined by petrographic, X-ray, and chemical means in order to determine exactly which phosphate phases were present. A subsequent evaluation which includes additional iron and aluminium phosphates has been reported [19].

MATERIALS AND METHODS

Preparation of phosphates

Monocalcium phosphate monohydrate, $Ca(H_2PO_4)_2 \cdot H_2O$, was used as a reference source of phosphorus. It was prepared by recrystallization of analytical-grade reagent in phosphoric acid and dried with acetone.

Colloidal ferric phosphates having two different P : Fe ratios were prepared by the precipitation method of Cate *et al.* [3] For the high-P sample, the precipitate was washed 3 times by decantation and filtration, the final filtrate having a pH of 2.5. After drying in a vacuum desiccator for 4 days, the solids had a P : Fe mole ratio of 1 : 1.13 and contained 25.0% Fe and 48.2% PO_4. When heated to 100°C, the sample lost 16.3% moisture. The particles had a diameter of approximately 0.05μ, a specific surface of 21.4 m^2/g as determined by glycerol absorption, and were amorphous to X-ray.

The low-P sample was prepared similarly, except that the washing and decanting procedure was continued for a 30-day period, at which time the final wash water had a pH of 4.2. The filtered solids, after drying in a vacuum desiccator at room temperature, had a P : Fe mole ratio of 0.98 and contained 23.8% Fe and 39.9% PO_4. Upon heating to 100°C, the sample lost 25.4% moisture. The solid particles were approximately 0.05μ in diameter, had a specific surface of 19.2 m^2/g, and were amorphous to X-ray.

Hydrogen ammonium iron phosphate, $H_8NH_4Fe_3(PO_4)_6 \cdot 6H_2O$, was a fine pink powder consisting of crystals and massive aggregates varying in particle size from 1 to 75μ. It contained 2.10% NH_4, 18.9% Fe, and 65.3% PO_4 and corresponded closely to the expected theoretical composition. The bulk phase was identified optically and by X-ray as Haseman's Group 5, Product K [11]. Only a trace of an unknown secondary phase was present. This phase had a refractive index above 1.70 and was undetected by X-ray. The method of preparation was similar to that reported for the analogous potassium ferric phosphate [19], except that equivalent quantities of NH_4 were used in place of K.

Strengite, $FePO_4 \cdot 2H_2O$, was prepared by the method of Cate *et al.* [3] and was identical to their material. It consisted of a single crystalline phase crystallized as rosettes, whose crystallographic and optical properties agreed with published values. Analyses showed 29.5% Fe and 50.8% PO_4. The

average particle diameter as determined by glycerol adsorption was 2.3μ, corresponding to a specific surface of 0.88 m^2/g.

Fluorapatite, $Ca_{10}(PO_4)_6F_2$, was prepared according to the method of Egan *et al.* [6] and contained 39.8% Ca, 56.6% PO_4, and 3.65% F. Its X-ray and crystallographic properties checked with published values and indicated no extraneous phases. The average particle diameter as measured by subsieve sizing was 13.5μ, corresponding to a specific surface area of 0.14 m^2/g.

Soils

The soils used in this study were (1) a Hartsells fine sandy loam, pH 4.9, (2) the same soil limed to pH 6.5, and (3) the same soil limed to pH 7.5, the pH measurements being made from a 1 : 1 soil—water suspension. The lime was supplied as a 3 : 1 weight mixture of $CaCO_3$ and $MgCO_3$, after which the soils were alternately wetted, dried, and mixed for a period of 30 days prior to cropping.

Fertilizer applications

Each of the phosphates was ground to pass a 325-mesh screen. Sufficient quantities to supply 50, 200, and 1000 mg of P were mixed with 3-kg lots of soil and placed in plastic-lined 3-liter tin cans. All treatments, including a no-P check, were replicated 3 times. Trace elements were supplied in the form of finely divided agricultural frits at the rate of 225 mg per can. N (100 mg) and K (100 mg) were added to each can in the form of NH_4NO_3 and KNO_3 at thinning time, and thereafter as needed to prevent N and K from limiting growth.

Calculations of relative availability coefficients

Relative availability coefficients were calculated from the Mitscherlich relationship

$$cx = \log \frac{A - Y_o}{A - Y_x}$$

where c = availability coefficient, x = quantity of fertilizer added, A = limiting yield of phosphorus with increasing x, Y_o = check yield, and Y_x = treatment yield. Values of A suitable to each crop and each soil were chosen so that a plot of the values of $\log (A - Y_x)$ against x gave a straight-line relationship for the MCP treatments similar to the procedure used by Bouldin and Sample [2]. The c values for each of the experimental treatments were then calculated, averaged for the 3 application rates and expressed as percentages of the corresponding c values for monocalcium phosphate (MCP), *e.g.*, $\frac{c_{\text{test source}}}{c_{\text{MCP}}} \times 100 =$ relative availability.

RESULTS

As shown in Table 1, uptake of phosphorus by corn increased markedly with increase in amount of monocalcium phosphate applied to the Hartsells fine sandy loam. The uptake of phosphorus ranged from an average of 3.85 mg of P per pot for no applied P to a maximum of 39 mg of P per pot in the third crop for the 1000-mg application of soil limed to pH 6.5. The lowest yields from MCP were obtained in the second crop on the unlimed and heavily limed soils.

TABLE 1

Uptake of phosphorus by three crops of corn from Hartsells fine sandy loam receiving additions of iron phosphates and lime

ertilizer source	mg P/pot	Soil pH 4.9			Soil pH 6.5			Soil pH 7.6		
		Crop			Crop			Crop		
		1	2	3	1	2	3	1	2	3
. . . . .	0	3.97	3.48	3.49	4.21	3.33	6.83	3.48	2.60	3.23
$_2PO_4)_2 \cdot H_2O$	50	7.00	5.12	6.28	6.21	10.5	8.26	3.92	3.87	6.50
CP)	200	14.5	12.3	14.4	11.8	21.7	19.6	6.65	8.11	17.8
	1000	27.2	19.8	27.8	27.4	30.3	39.0	26.3	20.7	28.7
dal Fe–P (High P)	50	5.12	4.89	5.97	5.11	6.87	10.4	3.96	2.64	5.54
	200	8.28	8.39	13.0	10.6	20.8	21.8	5.54	8.22	17.5
	1000	14.1	16.6	25.5	16.1	26.9	33.9	13.9	22.1	28.6
dal Fe–P (Low P)	50	5.28	3.74	4.16	5.34	7.41	9.37	3.25	3.29	5.94
	200	7.27	8.29	14.4	10.8	18.5	18.5	5.84	6.89	16.6
	1000	13.7	15.7	21.5	15.4	28.2	32.7	10.3	21.8	27.1
$H_4Fe_3(PO_4)_6 \cdot 6H_2O$	50	5.30	4.24	5.85	4.90	5.48	9.17	3.67	2.78	5.61
$-NH_4$–Fe–P) . .	200	8.46	8.98	16.1	5.38	7.95	15.2	3.56	2.98	12.4
	1000	17.4	16.4	26.5	11.6	22.0	27.6	7.60	22.3	24.4
$_4 \cdot 2H_2O$(Strengite)	50	5.09	3.24	4.48	4.17	4.06	6.97	3.52	2.51	3.07
	200	4.45	3.35	4.45	3.90	4.39	6.08	3.24	3.71	4.41
	1000	4.67	2.65	3.71	4.55	3.73	6.74	3.39	3.09	12.1
$PO_4)_6F_2$	50	4.61	2.74	4.14	5.04	3.61	6.26	3.16	2.65	3.36
uorapatite) . . .	200	4.57	3.17	3.93	4.27	3.21	7.82	3.24	2.92	3.46
	1000	4.28	2.84	3.75	4.18	3.80	7.07	3.31	2.93	3.64

Of the iron phosphates, only the two colloidal ferric phosphates and hydrogen ammonium ferric phosphate can be considered available sources. Strengite showed very little response and fluorapatite none. The high-P colloidal phosphate gave slightly greater phosphorus yields than the low-P sample; otherwise, the two colloidal materials behaved very similarly.

On the acid soil, MCP was consistently more effective than the available iron phosphates for the first and second crops. By the time of the third crop, the yields were more nearly equal. Liming to pH 6.5 increased the availability of the two colloidal iron phosphates but affected the average uptake of phosphorus from the acid ammonium ferric phosphate very little. Liming to pH 7.6 gave somewhat more variable responses. The uptake of phosphorus during the first crop was considerably reduced, particularly for the acid ammonium ferric phosphate and the lower rates of MCP. In the third crop all of the iron phosphates, as well as MCP, gave increased phosphorus responses.

The availabilities of the colloidal ferric phosphates (taken together) and the acid ammonium ferric phosphates for each crop and soil are compared to that of MCP in Figure 1. The relative availability coefficients plotted in this diagram were calculated from the Mitscherlich equation as described previously, and represent the effectiveness of a unit of each fertilizer source in producing the same uptake of P as obtained from MCP under comparable conditions. The marked superiority of MCP over the iron phosphates during the first crop was reduced in subsequent croppings. On the

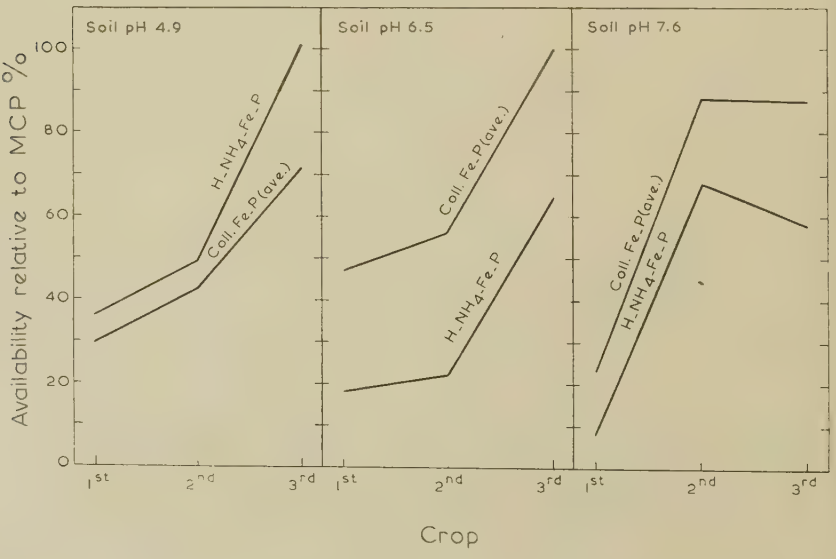


Fig. 1. The effect of soil pH and cropping on the relative effectiveness of two iron phosphates compared to MCP as 100.

unlimed soil, hydrogen ammonium ferric phosphate increased from 36 per cent the efficiency of MCP in the first crop to 101 per cent by the third crop, and exceeded the efficiency of the colloidal phosphates. In the soil at pH 6.5, the colloidal phosphates increased from 47 per cent the efficiency on MCP in the first crop to 100 per cent in the third. The hydrogen ammonium ferric phosphate was relatively much less available. At pH 7.6 the iron phosphates were extremely low in availability in the first crop but improved markedly in the second crop.

The total uptake of phosphorus by the 3 crops from each source applied at the 1000-mg rate are summarized in Figure 2. The uptake from MCP on the 3 soils averaged 82.4 mg of P per pot, as compared to 11.4 mg of P for the check yields. The colloidal phosphates and hydrogen ammonium ferric phosphate gave phosphorus responses slightly below those of MCP. The colloidal phosphates were most available at pH 6.5, as was MCP, while hydrogen ammonium ferric phosphate was affected much less by liming. Strengite was

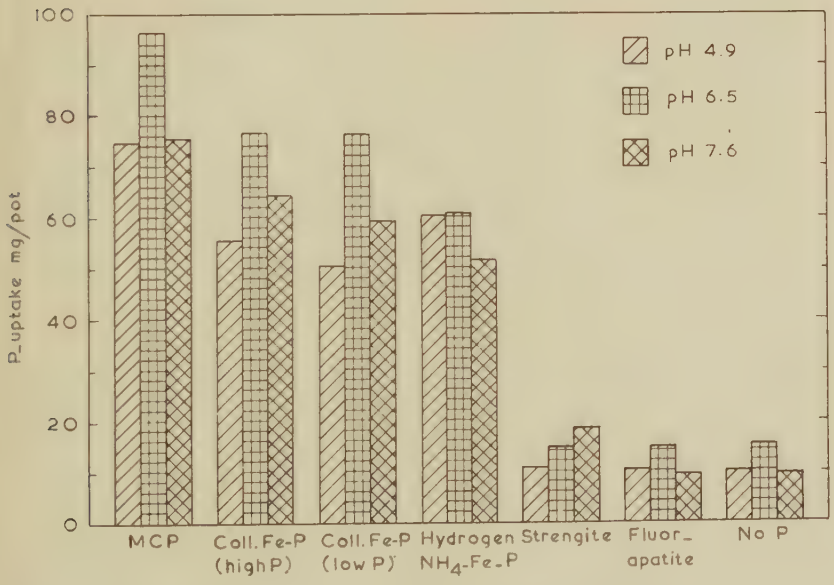


Fig. 2. Cumulative uptake of P by three successive crops of corn from Hartsells soil limed and fertilized with various iron phosphates at the rate of 1000 mg P/pot.

completely unavailable in the acid soil and showed only slight response at pH 7.6. As seen from Table 1, this response occurred mostly in the third crop. The fluorapatite was completely unavailable at all pH levels.

DISCUSSION

The phosphate materials of the present study were finely ground (−325 mesh) and mixed thoroughly throughout the soil. The large application rate (equivalent to 1500 pounds P_2O_5 per acre) was included in order to provide maximum opportunity for measuring any possible differences in availability of these relatively insoluble phosphorus compounds.

Colloidal ferric phosphates similar to those tested here are known to form initially during the reaction in soils with more soluble phosphates such as monocalcium phosphate [14]. Similar precipitation of colloidal ferric phosphates were noted in the reactions of dicalcium phosphate dihydrate with iron oxides [17]. The present results indicate that colloidal ferric phosphate is partially available to plants. The observed increase in availability with cropping could be accounted for by a gradual enlargement of the soil–fertilizer reaction zone surrounding each of the phosphate particles. Colloidal ferric phosphate is only slightly soluble, particularly in acid soils, hence the phosphate level in solution remains low and the reaction zone would spread slowly. The colloidal phosphates increased in availability with liming. This is explainable on the basis that the greater dissolution occurred at higher pH levels due to greater precipitation of ferric oxides with the concomitant release of phosphorus [5 15].

The hydrogen ammonium ferric phosphate, $H_8NH_4Fe_3(PO_4)_8 \cdot 6H_2O$, is representative of phosphate reaction products known to precipitate in the initially acid environment of fertilizer granules [14]. The present study indicates that reaction products of this type also provide available P for plants. This compound is known to hydrolyze under pH conditions encountered in soils. The extent of hydrolysis increases with rise in pH. The more erratic uptake of P from this compound upon liming was likely due to the coatings of ferric oxide which are precipitated on its surface during dissolution and hydrolysis. In time these coatings may become so extensive that

the initial material becomes isolated and further release of phosphorus is prevented.

Strengite is one of several known basic iron phosphates that may eventually form in soils. Our present knowledge of its solubility [5 15] indicates that a very low phosphate concentration can be maintained by this phosphate source, especially in acid soils. Its rate of solution also is known to be extremely low [13]. The present results lead to the conclusion that strengite is unavailable to plants in acid soils and slowly available even at pH 7.6. The effect of surface area on the availability of strengite may be an important factor in determining its availability to plants and needs further investigation.

The fluorapatite material included in the present study as an insoluble calcium phosphate gave no phosphorus response, and when precipitated in soils, would likely persist as such for a long time.

SUMMARY

Two well-characterized crystalline ferric phosphates, two colloidal ferric phosphates, and fluorapatite were tested under greenhouse conditions as sources of phosphorus for corn over a 3-cropping period. The selected compounds are representative of those expected to form in soils as reaction products from more soluble phosphate fertilizers.

Strengite, $FePO_4 \cdot 2H_2O$, was completely unavailable in acid soils and gave only a slight phosphorus response on soils limed to pH 7.6. Uptake of P from hydrogen ammonium ferric phosphate, $H_8NH_4Fe_3(PO_4)_8 \cdot 6H_2O$, was approximately 70 per cent that from MCP, and increased with cropping.

The colloidal ferric phosphates were approximately 78 per cent as available as MCP and became more available with liming and cropping. In the soil limed to pH 6.5, their effectiveness increased from 47 per cent that of MCP in the first crop to 100 per cent as effective by the third crop.

Fluorapatite, included as an insoluble calcium phosphate source, was completely unavailable.

ACKNOWLEDGEMENTS

Appreciation is expressed to Messrs. E. O. Huffman, W. E. Cate, E. L. Gurney, M. E. Deming, J. R. Lehr, J. P. Smith, and A. W. Frazier, of the Fundamental Research Branch, for their cooperation in the preparation and characterization of the materials used in this study.

Received June 2, 1960

REFERENCES

1 Bartholomew, R. P. and Jacob, K. D., Availability of iron, aluminum, and other phosphates. J. Assoc. Off. Agr. Chem. **16**, 598–611 (1933).

2 Bouldin, D. R. and Sample, E. C., Calcium phosphate fertilizers: III. The effect of surface area on the availability coefficients of the dicalcium phosphates. Soil Sci. Soc. Am. Proc. **23**, 276–280 (1959).

3 Cate, W. E., Huffman, E. O., and Deming, M. E., Preparation of crystalline ferric phosphates. Soil Sci. **88**, 130–132 (1959).

4 Chang, S. C. and Jackson, M. L., Soil phosphorus fractions in some representative soils. J. Soil Sci. **9**, 109–119 (1958).

5 Chang, S. C. and Jackson, M. L., Solubility product of iron phosphate. Soil Sci. Soc. Am. Proc. **21**, 265–269 (1957).

6 Egan, E. P., Jr., Wakefield, Z. T., and Elmore, K. L., Thermodynamic properties of fluorapatite, 15 to 1600°K. J. Am. Chem. Soc. **73**, 5581–5582 (1951).

7 Fujiwara, A., Chemical constitution and availability of iron and aluminum phosphates: I. Studies on the constitutions and manurial effects of scarcely-soluble phosphates. Tohoku J. Agr. Research **1**, 129–141 (1950).

8 Fujiwara, A., Chemical constitution and availability of iron, aluminum, and calcium phosphates reprecipitated from solution: II. Studies on the constitutions and manurial effects of scarcely soluble phosphates. Tohoku J. Agr. Research **1**, 143–149 (1950).

9 Fujiwara, A., Effects of heating and dehydration on the constitution and availability of scarcely soluble phosphates: III. Studies on the constitutions and manurial effects of scracely soluble phosphates. Tohoku J. Agr. Research **1**, 224–228 (1950).

10 Haseman, J. F., Brown, E. H., and Whitt, C. D., Some reactions of phosphate with clays and hydrous oxides of iron and aluminum. Soil Sci. **70**, 257–271 (1950).

11 Haseman, J. F., Lehr, J. R., and Smith, J. P., Mineralogical character of some iron and aluminum phosphates containing potassium and ammonium. Soil Sci. Soc. Am. Proc. **15**, 76–84 (1951).

12 Hsu, P. H. and Jackson, M. L., Inorganic phosphate transformations by chemical weathering in soils as influenced by pH. Soil Sci. **90**, 16–24 (1960).

13 Huffman, E. O., Cate, W. E., and Deming, M. E., Rates and mechanisms of dissolution of some ferric phosphates. Soil Sci. **90**, 8–15 (1960).

14 Lindsay, W. L. and Stephenson, H. F., Nature of the reactions of monocalcium phosphate monohydrate with soils: IV. Repeated reactions with metastable triple-point solution. Soil Sci. Soc. Am. Proc. **23**, 342–345 (1959).

15 Lindsay, W. L. and Moreno, E. C., Phosphate phase equilibria in soils. Soil Sci. Soc. Am. Proc. **24**, 177–182 (1960).

16 Marais, J. S., The comparative agricultural value of insoluble mineral phosphates of aluminum, iron, and calcium. Soil Sci. **13**, 355–410 (1922).

17 Moreno, E. C., Lindsay, W. L., and Osborn, G., Reactions of dicalcium phosphate dihydrate in soils. Soil Sci. **90**, 58–68 (1960).

18 Sannikova, N. M., Iron and aluminum phosphates as sources of phosphoric acid for plants. Trans. Sci. Inst. Fertilizers Insectofungicides (USSR) **141**, 70–76 (1938).

19 Taylor, A. W., Gurney, E. L., and Lindsay, W. L., An evaluation of some iron and aluminum phosphates as sources of phosphate for plants. Soil Sci. **90**, 25–31 (1960).

20 Truog, E., The utilization of phosphates by agriculture crops, including a new theory regarding the feeding power of plants. Wisconsin Agr. Expt. Sta. Research Bull. **41** (1916).

Plant and Soil XIV, no. 2 April 1961

PHYSIOLOGICAL FACTORS LIMITING GROWTH AND YIELD OF *ORYZA SATIVA* UNDER UNFLOODED CONDITIONS

by S. T. SENEWIRATNE * and D. S. MIKKELSEN

Department of Agronomy, University of California

INTRODUCTION

The origin of rice culture dates back over 5000 years and is lost in antiquity. With passing centuries rice has acquired a wide diversity of cultural methods. These vary from unflooded or upland culture, where the soil is kept moist and aerated, to flooded or lowland culture, where the crop is grown under partial or complete submergence. The growth of all varieties of rice is generally favored by flooding and yields have been increased as much as 60 per cent over unflooded culture [14]. It appears that no variety of rice, either upland or lowland types, has the capacity to overcome the disadvantages encountered when grown in a well-aerated soil.

Much has been written to explain why rice, a morphologically non-aquatic plant, should germinate and flourish under flooded conditions. In contrast, no substantial effort has been made to investigate the process or processes limiting the growth of rice under unflooded conditions. Several benefits of flooding have been determined, but the physiological basis for the superior growth of rice is not agreed upon. Available evidence indicates that rice benefits from an abundance of water, yet does not have a water requirement greater than other common field crops. Standing water, apart from weed control and micro-climate regulation, appears to have no beneficial physiological role. The flood water may supply some nutrients to the crop, and may enhance nitrogen

* Present address: University of Ceylon, Peradeniya, Ceylon.

fixation by blue-green algae and other organisms. These may be important factors in areas where soil fertility limits production. Soil submergence also brings about changed oxidation–reduction conditions which alters the soil gases, the form and availability of plant nutrients, and other soil constituents.

Evidence suggests that variations in the availability and form of nitrogen, iron, manganese, and phosphorus, either singly or as a result of some interactions, may determine the physiological basis for the superiority of flooded rice culture. The approach of experiments in this investigation has been to evaluate the effect of these variables and some of their interactions on the mineral nutrition and related physiology of the rice plant.

METHODS

The experiments reported in this study were conducted under typical field conditions and where greater control was necessary, under greenhouse conditions. The procedures used were designed to critically evaluate: (1) the effect of flooded and unflooded culture on the growth and nutrient uptake of *Oryza sativa, japonica* var. Caloro and, (2) the various interrelations of nitrogen, phosphorus, iron, and manganese in controlled nutrient cultures.

Field experiments

Rice was grown on a Stockton clay soil, pH 4.7, in both unflooded and flooded culture. In the unflooded treatment, seed was drilled 1 inch deep in a prepared seedbed in rows 6 inches apart and irrigated. The flooded treatment was sown into standing water after land preparation and flooding. The water level in this treatment was maintained at a height of 6″ throughout the growing season. The seeding rate was 100 pounds per acre in both treatments.

Whole-plant samples were collected at weekly intervals during most of the growing season. Plants were collected at random in the plots between 8 and 10 a.m. The collected materials were carefully washed, dried in a forced-air oven at 70°C and then saved for growth measurements. For purposes of discussion, the coleoptile was considered the first leaf and the bladeless leaf sheath, the second leaf. All other leaves were numbered from 3 to 15 according to the time of exertion, leaf 15 being the "flag" leaf. After elongation had taken place, the culm of the main stem was separated, internode by internode. Length and weight of leaves, culms, roots, and inflorescence, if any, were recorded for the main stems.

Plant height and root length were measured on fresh material representing 15 plants per treatment. Leaf area was determined on fresh tissue by taking prints on sensitized paper. These prints were cut out and weighed,

and the area of the leaves was then calculated. Moisture content was determined on a separate, freshly harvested sample.

The progress of tillering was studied on 50 plants taken at random from each treatment. Plants were marked and each tiller was tagged as it was produced.

Plants materials were dried and analyzed for nitrogen, by the Kjeldahl method, and phosphorus, sulfur, iron, magnesium, and manganese by methods described by Toth *et al.* [26]

Plant materials collected from the field and soil treatments in large tubs maintained in the greenhouse were sampled 45, 60, 80, and 110 days, respectively, after sowing. A composite sample of blades from the 3rd and 4th leaves, as well as culms, were frozen in dry ice and maintained at 17°C for a short period prior to analysis. The frozen tissues, separated before freezing into 2- and 5-gram lots, were used for the determination of "soluble" iron and manganese by methods described by Sommers and Shive [24], for catalase activity as determined by Haskins [8], and peroxide, as outlined by Siegel and Galston [6]. Assays of the samples for 3-indoleacetic acid were not successful because of interfering substances in the tissue extracts.

Nutrient-culture experiments

The nutrient-culture experiments were conducted with both ammonium and nitrate as a source of nitrogen. For convenience they are referred to as the ammonium and nitrate cultures. The nitrogen cultures were made up from either $Mg(NO_3)_2$ or $(NH_4)_2SO_4$ with additional $CaCl_2$, $MgSO_4$ and a micro-nutrient supplement. The nutrient solutions were made with distilled water and had the following composition at full strength.

Composition of nutrient solutions (Concentration in ppm)											
Nitrogen series	N	K	Mg	Ca	S	Cl	Mn	Mo	B	Zn	Cu
NH_4–N	56	23	48	20	128	35	0.5	0.01	0.5	0.05	0.02
NO_3–N	56	23	73	20	32	35	0.5	0.01	0.5	0.05	0.02

It was not possible to use identical anion and cation concentrations in the two series of cultures. Magnesium and sulfur were considered less critical elements in this study and they were varied.

With each of these ammonium- and nitrate-nitrogen series, iron was varied using ferrous sulfate. The levels of iron used were: 0, 0.005, 0.010, 0.030, 0.060, 0.100, and 0.200 ppm; phosphorus was maintained constant at 9 ppm in these cultures. Similarly with each of the nitrogen sources, a phosphorus series was run using potassium dihydrogen phosphate to supply phosphorus levels of: 0, 0.10, 0.50, 1.00, 2.00, and 3.00 ppm; iron was maintained constant at 1 ppm in this series.

Reagent-grade chemicals were used throughout and no effort was made to purify these salts. The maximum amounts of iron and phosphorus carried

as impurities were determined to be 0.0006 ppm and 0.00012 ppm, respectively.

Young rice plants do not tolerate high salts concentrations, therefore the seedling plants were started in culture solution maintained at quarter strength for the first week, half strength the second week and full strength thereafter.

All solutions were changed twice weekly throughout the experiment, and the pH of the solutions were maintained between pH 3.7 and 4.0. No change in the form of nitrogen in the culture solution was detected from one change of solution to the next.

Harvested plant materials were analyzed after drying by the methods described earlier.

EXPERIMENTAL RESULTS

Influence of cultural treatments on growth

Observations made by earlier investigators on the performance of rice grown under the two cultural conditions being evaluated, amounted to estimates of yield in most cases. Since yield integrates all the factors affecting the crop any attempt to analyze the factors contributing to final yield must include a detailed study of the development of the plant. Further, it was conceivable that detailed growth studies could help direct attention towards the basic physiological relations responsible for the growth differences. To make the discussion of growth developments more meaningful, a brief calendar of growth is presented in Table 1.

Results on seedling, tillering, and post-tillering stages of growth will be discussed separately.

Seedling stages. Differences in seedling growth due to treatment were evident soon after germination. Seven days after sowing, the plants grown under unflooded conditions appeared robust, weighed more, were taller, and had more roots. After 14 days the plants under flooded conditions produced enhanced shoot growth but had restricted root growth. The accelerated shoot growth and poor root development are explained on an auxin basis as influenced by standing water during germination [9]. Between the 11th and 14th day, the plants in the flooded treatments exerted their leaves through the water. At the same time it speeded up root elongation which is likewise attributed to an auxin relationship.

Apart from plant height, the seedling growth under unflooded

TABLE 1

Calendar of growth events		
Age of plants in days	Flooded rice	Unflooded rice
19	Plants through the water; leaves lying flat on water surface	Robust plants
26	First leaves standing above water	Tillering begun
33	Tillering just begun. Plants light green	50 per cent tillered. Plants dark green
40	Plants tillered, and darker green than before	Plants dark green and well tillered
54	Broad leaves as compared to unflooded. Roots thick and relatively unbranched	Narrow leaves. Roots branched and not thick
61	Plants showing new root system on surface. Plants better grown than these in other treatments	No new root system observed
68	New root system developing fast	No new root system
75	Plants green, healthy appearance. Culm elongating. Plants taller	Plants small, but green, healthy appearance. No elongation of culm
82	Two nodes of the culm elongated. Inflorescence visible	No internodes elongated. Inflorescence smaller. Plants producing small tillers
89	Inflorescence well developed and in early boot stage	Little development of inflorescence
96	Plant in heavy boot stage. Flag leaf out	Leaves distinctly yellowing. Heads still small
103	Inflorescence about to be exerted. Flag leaf dropped	Leaves continue to yellow. Inflorescence developing
110	Inflorescence exerted	Inflorescence in boot
116		Inflorescence not exerted
123		Inflorescence exerted in some plants

conditions made vigorous early growth and produced a larger amount of dry matter and a materially increased leaf area. From these studies it is evident that unflooded culture favors seedling growth. As such, if subsequent environmental conditions are equal, these more vigorous seedlings would be expected to produce better growth and yields.

Tillering phase. Tillering commences soon after seedling establishment. The importance attached to tillering, arises from the

fact that it is positively correlated with yield, as shown by Rao [19] and others.

The first observable differences between cultural treatments with regard to tiller production, was the age at which the plants tillered. The plants grown under unflooded conditions tillered one week ahead of those grown flooded. The "grand period" of tillering was short-lived and reached its peak for both treatments about the 49th day. The flooded treatment produced an average of 4 tillers in addition to the main stem. In comparison, the unflooded treatments produced only 3 tillers per plant.

The plant samples collected were examined to determine the locus of tiller production. Tillers originated from a lower node in the plants grown under well-drained soil conditions. The lowest tillers on the plants grown under unflooded conditions originated from the axil of the third leaf. In contrast, the plants in the flooded treatment started tillering from the fifth node and on upwards. This confirms the work of Sekiya [22] with regard to change in tillering locus on flooding.

The high correlation between tiller order and its liability to die or remain unproductive has been reported by Rhind and Thein [21]. The plants collected from both treatments had only first-order tillers. There was, however, an appreciable difference in the rates of productive to unproductive or dead tillers between treatments. The flooded treatment produced an average of 21 per cent unproductive tillers as compared with 39 per cent for the unflooded. The tendency for the latter treatment to remain small was conspicuous.

Unflooded culture appeared to exert a growth-restraining effect on tiller development. Data showing changes in dry weight of tillers during the period of 95 to 160 days shows the increase in flooded to be almost double that of unflooded plants. These large differences in dry-matter production indicate that a physiological mechanism may be involved.

Post-tillering to harvest. The title post-tillering is not meant to indicate a total absence of tiller production but denotes the phase covering 100 days commencing after the "grand period" of tillering. This period includes over-lapping growth stages, involving the change from vegetative to the generative phase, the development and exertion of the inflorescence, ripening of the grain and senescence of the plant as a whole.

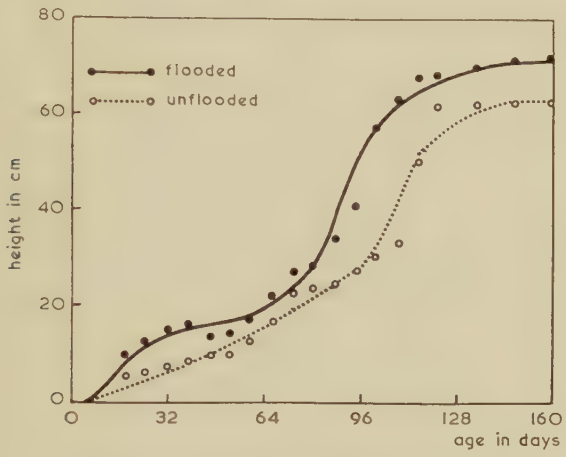


Fig. 1. Seasonal increase in plant height.

Growth in plant height is presented in Figure 1. The early rapid growth in height of the plants grown under flooded conditions was associated with emergence through water. The subsequent rapid elongation – about the 96th day, 14 days ahead of the unflooded plants – was due to culm extension a feature which will be discussed in greater detail.

Increase in leaf growth deserves close study, as it is the main avenue through which the plant elaborates carbohydrates and thus determines yield. However, the rice plant consists of a colony of tillers, some productive, others not, and it is a cumbersome unit for detailed examination. Since the main stem is less liable to variation from plant to plant, it was selected for study in making further comparisons between treatments. Detailed growth studies were made on each leaf at weekly intervals, however, only appropriate data on the 7th, 10th, and 13th leaves will be presented, since they were considered representative.

Under the consideration on seedling growth, the early advantage of the unflooded treatment in increasing leaf weight and leaf area was stressed. This is confirmed by this data, but the advantage lasted only until about the 39th day. From this day a change in relative treatment response was noticed, the flooded treatment showing quicker leaf weight gains. After reaching a maximum leaf weight about the 102nd day, a decline set in which apparently

resulted from the translocation of elaborated food material out of the leaves. In comparison, the plants in the unflooded treatment did not reach maximum leaf weight until the 116th day and did not seem to translocate elaborated food out of the leaves until after the 149th day. The main difference appeared to be a restrained growth effect on the plants in the unflooded treatment.

Examination of the time of exertion and senescence of each leaf on the main stem shows that up until the 32nd day, or the time when the 8th leaf was exerted, the unflooded treatment either produced leaves ahead of the flooded treatment, or they both exerted a new leaf during the same week. From then on, the flooded treatment produced leaves faster than the unflooded.

The effect of treatments on leaf area is interesting. Using three representative leaves, it was seen that leaf areas of plants grown under flooded conditions, was greater than of the unflooded. The leaf areas of three selected leaves are shown in Figure 2. During the period when the plants were 50 to 90 days old there was a difference of 20 to 34.5 cm^2 in leaf area per main stem between unflooded and flooded treatments, respectively. When this difference is further weighed with the consideration that the longest daylengths in the year coincided with this period, one cause of depressed dry-matter production and yields of unflooded rice can easily be visualized.

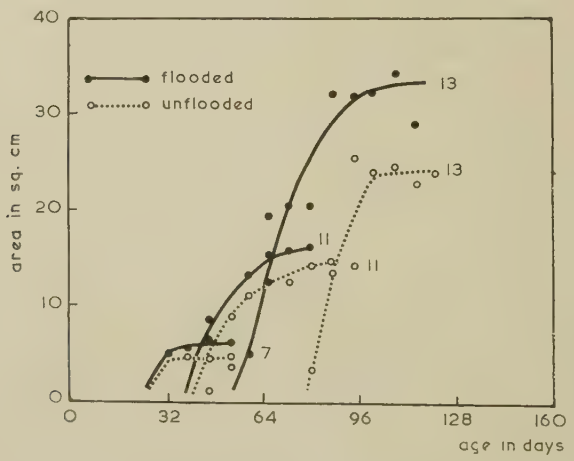


Fig. 2. Seasonal increase of area of leaves in sq. cm.

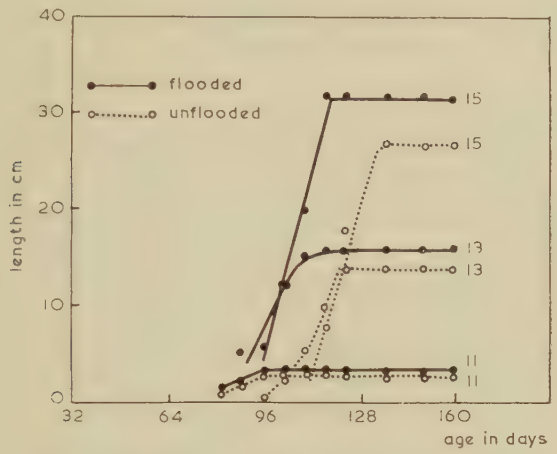


Fig. 3. Seasonal increase in length of internodes.

Internode elongation in the different culture treatments are shown in Figure 3. Internode 11, produced little growth while internode 13 and 15 both showed appreciable elongation. These internodes not only indicated a treatment difference in regard to time of elongation, but also in respect to final length. Here again depressed and delayed growth were observed in the unflooded treatments.

The final result of the delayed rate of elongation of the culm in the unflooded treatment, was the later flowering of these plants.

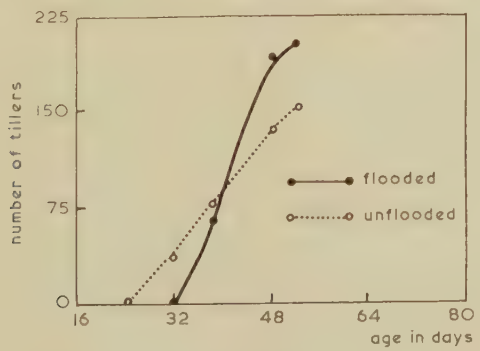


Fig. 4. Seasonal increase in number of tillers. (Based on a sample of 50 plants).

This difference in flowering amounted to about 14 days, some plants being a little further ahead of others.

The cumulative effect of poor tiller growth, shown in Figure 4 along with slow leaf exertion and smaller leaf area and delayed flowering, resulted in fewer and less plump grain on plants in the unflooded treatments. The yield of grain on the unflooded treatment was only 52.6 per cent of the flooded, which is in fair agreement with the yield data reported by Matsuo [14] who obtained 60 per cent for the same comparison. In general, yield reduction was caused by fewer grain bearing tillers, less grain per tiller and smaller kernel size.

Another interesting difference was observed in regard to the moisture content of the plants. The moisture content increased with age as similarly observed by Suzuki [25]. The important difference arises from a comparison of moisture content in plants between treatments. An average of 4 to 5 per cent higher moisture in the plants grown under flooded conditions was observed from the 46th to the 95th day. Apart from the physiological significance of this result, the studies of Murato *et al.* [15] show that increased moisture content was positively correlated with photosynthetic activity. This would thus be added advantage for this treatment from the point of view of carbohydrates elaboration.

Influence of cultural treatment on nutrient uptake

Composite sample of leaves representing every leaf position on the main stem were analyzed separately for certain mineral constituents. Only data from leaves 7, 10, and 13 are discussed since they adequately reflect the nutritional status of the treated plants.

The general trend for all the leaves showed a gradual decline in nitrogen, phosphorus, potassium, sulfur, and magnesium composition with increased maturity. This decline during early development of the leaves was associated with dilution as a result of carbohydrate elaboration and at later stages with translocation of material out of the leaves with approaching senesence. These elements in leaf 7 decreased earlier in the flooded treatment. This difference is due to the shorter life period of leaves on plants grown under flooded conditions and applies also for leaves 10 and 13. The data obtained did not show significant differences in composition of the leaves of the two treatments and no recognized symptoms of deficiencies occurred.

The iron composition showed a gradual increase in the leaves as they matured, but no significant differences were noted between flooded and unflooded plants.

As in the case of iron, manganese accumulates in the tissues and the data revealed a very constant relationship in all leaves. Under flooded culture the manganese content of leaves started low and gradually increased with leaf age. The manganese content of leaves from unflooded plants likewise increased but to a much greater extent. This difference between treatments in total manganese content ranged from 250 to 773 per cent on plants growing in unflooded treatments, as compared with the flooded.

In analytical observations made thus far discussions have been concerned with total iron and manganese in the plant tissue. The active iron and manganese in plants are considered to be the fractions which are in solution within the cells, and as such may be more directly involved in physiological activities of the plants [24]. The active iron and manganese referred to here as the soluble fraction was obtained by pressure extraction. The fraction remaining in the press cake is referred to as the inactive or insoluble fraction. The analytical results obtained on leaves from plants grown flooded, unflooded, and unflooded with subsequent flooding are shown in Table 2.

The striking similarity in the soluble, insoluble, and total iron content of the plants regardless of treatment was evident from this study. A comparison between iron content in the insoluble and soluble fractions showed that there was nearly twice as much iron in the insoluble fraction as there was in the soluble fraction. The

TABLE 2

Soluble and insoluble iron and manganese fractions of leaves, from plants grown flooded, unflooded, and unflooded with subsequent flooding

Metal fraction	Per cent iron			Per cent manganese		
	Flooded	Unflooded + flooded	Unflooded	Flooded	Unflooded + flooded	Unflooded
Soluble fraction	.0018	.0016	.0018	.0044	.0212	.0375
Insoluble fraction	.0034	.0037	.0029	.0058	.0290	.0414
Total	.0052	.0053	.0047	.0102	.0502	.0789

proportion appeared to be a little less in the plants grown in the unflooded culture.

The results obtained on manganese content of both fractions showed a wide variation between treatments. The plants grown under flooded culture contained the lowest manganese in respect to both fractions. A treatment that was started unflooded and subsequently flooded was intermediate and the continuously unflooded treatment had the highest manganese content in both fractions. Regardless of treatment, in every instance the insoluble fraction contained more manganese. The ratio of manganese in the soluble as compared to the insoluble fraction was similar for all the treatments.

In the analysis of young leaves, older but green leaves, and chlorotic leaves, it is striking that there was little difference in the iron content of the soluble fraction. However, the insoluble fraction obtained from chlorotic leaves contained more iron than the green leaves.

The manganese content of young leaves was only a little higher in plants grown in the unflooded treatment. The soluble manganese fraction obtained from the older green leaves contained more than twice as much manganese as found in the young leaves. The chlorotic leaves contained by far the largest amounts of manganese in both fractions.

Nutrient culture experiments

One of the basic differences between flooded and unflooded rice culture is the form in which the plants absorb their nitrogen. Under oxidizing conditions prevailing in an unflooded soil, nitrate nitrogen is the main source of nitrogen available to plants. Under submergence, where nitrates are rapidly denitrified, ammoniacal nitrogen predominates. The ability of rice to utilize both forms of nitrogen has been amply demonstrated but conflicting data exist concerning the suitability of each form. It therefore seemed desirable to evaluate nitrogen source together with interactions of iron and phosphorus in these experiments.

The cultural solution experiments showed that plants grown in nitrate nitrogen cultures made better growth than plants in ammonium cultures when supplied with adequate iron and phosphorus.

Results of all the interactions of source of nitrogen, iron and

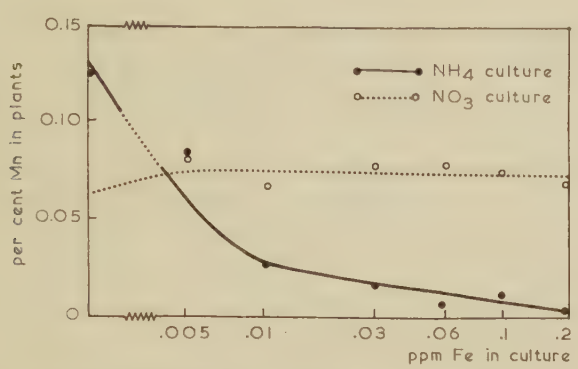


Fig. 5. Percentage manganese in plants as influenced by iron levels.

phosphorus cannot be discussed here. It is significant to note, however, the influence of iron levels in cultures of nitrate and ammonium-nitrogen on the accumulation of manganese. As shown in Figure 5, the plants grown in ammonium culture accumulated manganese when no iron was added. With increasing iron supply, the manganese levels in the tissue decreased steadily, to about 0.01 per cent with adequate iron. In the nitrate nitrogen cultures the level of iron supply had no influence on manganese content, within the range of iron levels used in this experiment. At adequate levels of iron, plants in nitrate cultures had 6 to 7 times as much manganese as those in ammonium cultures.

The response of plants to increasing phosphorus levels in nitrate and ammonium nitrogen cultures is shown in Figure 6. With

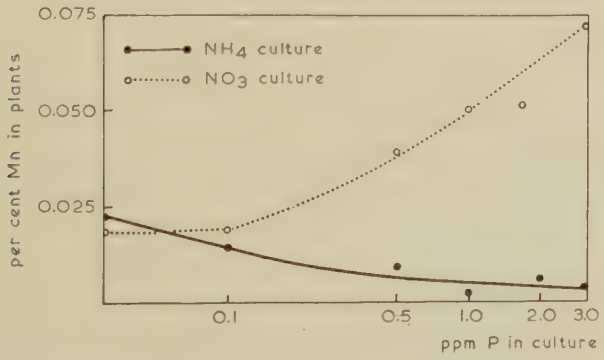


Fig. 6. Percentage manganese in plants as influenced by phosphorus levels.

ammonium nitrogen, the response of rice to increasing phosphorus was one of gradual depression of manganese content. At adequate phosphorus levels of 1 ppm or more the plants contained only traces of manganese. In sharp contrast, with nitrate nitrogen and increasing phosphorus supply, the manganese content increased likewise. Within the range of phosphorus levels studied from 0 to 3.0 ppm, the manganese content increased nearly 400 per cent.

In these solution culture experiments, variations in kind of nitrogen, phosphorus, iron, and manganese were evaluated. These systems exist under flooded and unflooded rice culture and offer additional understanding of rice nutrition, growth and ultimately yield effects. It has been demonstrated that the source of nitrogen interacting with iron and phosphorus help determine the manganese content of rice plants.

Since auxins could not be determined directly in extracts from plants grown under flooded and unflooded conditions, enzyme systems known to influence auxin metabolism in other plants were investigated. Inasmuch as catalase and peroxidase activity have been directly linked to iron and manganese variations, the activities of these systems in plant extracts were evaluated.

The data on variation in catalase activity between treatments are presented in Figure 7. The catalase activity measured from plant extracts from the flooded plants was greater than from the unflooded. In contrast the extract obtained from plants grown under unflooded conditions showed higher peroxidase activity (Figure 8). The significance of peroxidase and catalase variations between

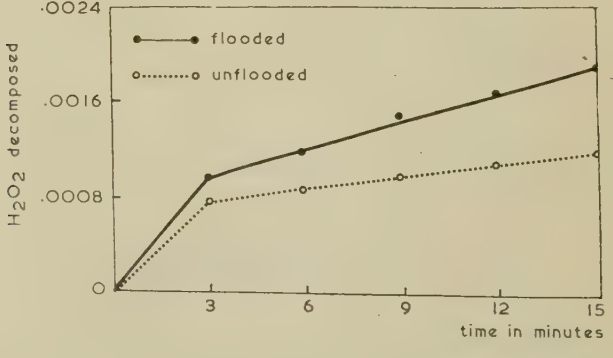


Fig. 7. Catalase activity of leaf extracts.

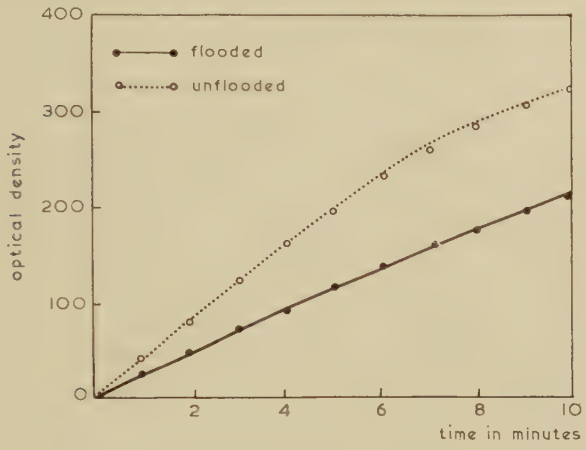


Fig. 8. Peroxidase activity of leaf extracts.

treatments, arising from high manganese accumulation, has been associated with auxin destruction in some plants [3 13]. These enzymes may also directly effect the growth response in rice. The physiological significance of these enzyme variations in relation to plant growth will be discussed subsequently.

DISCUSSION

Results of field and greenhouse experiments have shown both physiological and nutritional differences in rice grown under flooded and unflooded conditions. In evaluating the salient growth characteristics, it was shown that plants grown in unflooded culture were more robust, had a greater dry weight, and appeared in better tone than flooded plants until about 35 days after emergence. After this period, the unflooded plants appeared to suffer from some growth-retarding influence, even though soil moisture was kept near field capacity. The unflooded plants gradually became chlorotic, had a lower moisture content, tiller number was restricted, tiller development retarded, leaf area was reduced, and leaves were exerted at a slower rate. The appearance of macroscopic flower primordia occurred about the same time in both treatments, but a delay in internode elongation and flowering was observed in the unflooded treatment. Grain yields of the

unflooded rice showed a 52.6 per cent reduction as compared with the flooded treatment.

In interpreting these growth responses, the plant behaviors are suggestive of differences in auxin metabolism. The experimental verification of this observation however involved prohibitive difficulties in analytical determinations and interpretation of results. Some relationship can be developed however by relating the observed plant responses to known auxin effects obtained under controlled conditions.

Several of the growth responses observed in these experiments are possibly related to plant auxin metabolism. The depressed plant growth and leaf area (Figures 1 and 2), the lower number and poorer survival of tillers (Figure 4), delayed flowering and reduced number of grains per head are all well-known auxin effects on plant growth [2 7]. The low moisture content of the unflooded plants, in spite of their growth in adequate soil moisture, is an auxin effect observed by various investigators [17 23 27 28] and reviewed by Bonner and Bandurski [4]. The more rapid and extensive internode elongation observed under flooded culture (Figure 3) may be an auxin response similar to that obtained by Liverman [12] and Koneshi [10]. Evidence would indicate that rice grown under flooded conditions has a higher auxin level, and thus internode elongation is initiated earlier. Tillering in grasses is known to be influenced by low auxin levels [11].

The analogous evidence obtained from the growth observations and related auxin response supports the contention that plants grown under unflooded conditions probably contain low auxin levels. Such low levels of auxin could arise from either a subnormal capacity for auxin synthesis or enhance auxin destruction which does not contribute to the growth processes. Variations in auxin synthesis could be genetically controlled or influenced by nutrient relationships among other environmental factors. Since the same variety was used in these experiments, the factor of genetic variability can be eliminated.

The chemical composition of plants harvested from flooded and unflooded treatments were not essentially different in total nitrogen, potassium, phosphorus, magnesium, sulfur, or iron content. There was however a markedly higher manganese content of plants grown under unflooded conditions. The presence of 0.40 per cent manganese

was far in excess of values reported for rice grown under flooded conditions and also for other crops.

Since a pronounced interdependency of iron and manganese has been observed [30] and since it has been suggested that the poor growth of unflooded rice might be caused by iron deficiency [18], the iron – manganese inter-relationship was examined. Results presented in Table 2 did not reveal a constant iron – manganese relationship in the treated plants. The iron content of the soluble or "active" fraction and the insoluble fraction were nearly the same in all treatments but the manganese content varied widely. The iron – manganese ratios were 0.41 and 0.048 respectively for flooded and unflooded plants.

Green and chlorotic leaves from plants grown under unflooded conditions were analyzed to establish a basis for their physiological condition. No differences in iron content were observed but a high manganese content was associated with the symptoms of chlorosis. The chlorosis observed in the plants was unlike iron deficiency described [3, 29] and recognized locally. It seems unlikely that iron deficiency would occur in the presence of adequate levels of iron in the tissue. The possibility is not precluded however that some physiological mechanism might cause chlorosis under unflooded conditions, even in the presence of adequate iron within the plant.

Nutrient-culture experiments were conducted to evaluate the effects of ammonium and nitrate nitrogen, iron, phosphorus and some of their interactions on the mineral nutrition and related physiology of rice. Results indicated that rice could use both forms of nitrogen but that plants grown in nitrate cultures made better vegetative growth when supplied with adequate iron and phosphorus. It was shown (Figures 5 and 6) that plants supplied with ammonium nitrogen with adequate iron and phosphorus contained small amounts of manganese. Nitrate nitrogen, under similar conditions, favored the accumulation of manganese. These results agreed with data obtained from plants grown under field conditions. Rice utilizing nitrate nitrogen, such as occurs under unflooded, aerated soil conditions favors manganese accumulation. Flooded rice which uses principally ammonium nitrogen did not show manganese accumulation. This effect of nitrate nitrogen on manganese accumulation has been observed by Olsen [16]. Fredericksen [5] shows a reciprocal antagonistic effect of iron on manganese

in the presence of ammonium as a nitrogen source for plants.

Consideration of the growth responses observed under unflooded conditions as typical auxin effects, together with the unusually high manganese content of these plants suggests the possibilities of manganese-induced growth regulation. A relationship between manganese and the indoleacetic acid oxidase system, involving catalase and peroxidase activity has already been established [3 13] and reviewed recently by Ray [20]. They found that high manganese in plant tissue was associated with high peroxidase and low catalase activity. The indoleacetic acid oxidase system, responsible for the destruction of the most commonly found native auxin, is expressed [6] simply as:

$$H_2O_2 + IAA \xrightarrow{\text{peroxidase}} \text{oxidized IAA} + H_2O$$

From this reaction it can be visualized that if peroxide was not limiting and other conditions were favorable, the destruction of auxin would be directly related to peroxide availability. Similarly, if peroxide was not limiting, the rate of destruction would then be determined by peroxidase activity. Since the enzyme catalase is known to destroy peroxides, the final peroxide content in plant cells will be determined at least in part by catalase activity.

The catalase and peroxidase activities of plant extracts obtained from flooded and unflooded rice are shown in Figures 7, and 8, respectively. A depressed catalase activity was observed in extracts from plants grown under unflooded conditions, while the same plants had a higher peroxidase activity than flooded-culture rice.

Considering the effect of low catalase and high peroxidase activity of plants grown under unflooded conditions, accelerated auxin destruction would be expected. In contrast, the effect of high catalase and low peroxidase activity acting together would depress auxin destruction in flooded rice. These changed enzyme relations, resulting from variations in plant manganese content appear to create wide differences in auxin levels between treatments. These auxin affects, associated with foliar chlorosis, also the result of excessive manganese, appears to be the determining factors limiting the growth and yields of rice under unflooded cultural conditions.

SUMMARY

Rice grown under flooded conditions consistently produces better vegetative growth and higher grain yields than when grown in unflooded culture. Physiological and nutritional differences in rice grown under these two conditions were determined. Growth observations showed that plants under unflooded culture made an initial vigorous start, but soon showed poor tillering, depressed leaf growth, delayed flowering, low moisture content, foliar chlorosis, and 52.6 per cent lower yield than flooded plants.

Chemical analysis emphasized the higher manganese content of plants grown under unflooded culture with no significant differences in other elements. Plants grown in nutrient cultures and under field conditions gave evidence that nitrate nitrogen nutrition, as exists for plants under unflooded conditions, favored manganese accumulation.

Growth responses suggest differences in auxin metabolism. Since auxins could not be estimated directly, some factors affecting auxin degradation were investigated. It was found that plants grown under unflooded conditions had: 1) a low catalase activity, and: 2) a high peroxidase activity, which favor accelerated auxin degradation. It is proposed that high manganese levels in plants grown under unflooded conditions affects the indoleacetic acid oxidase mechanism resulting in retarded growth and depressed grain yields.

Received May 31, 1960. Revised draft November 28, 1960.

LITERATURE CITED

1 Aiyer, S. P., The role of minor elements in rice growing. Indian Farming **7**, 11–14 (1946).

2 Asana, R. D., Mani, V. S., and Vedprakash, The effect of auxins on the growth and yield of wheat. Physiol. Plantarum **8**, 279–287 (1955).

3 Bailey, L. F. and McHargue, J. S., Effect of boron, copper, manganese, and zinc on the enzyme activity of tomatoes and alfalfa plants grown in the greenhouse. Plant Physiol. **19**, 105–116 (1944).

4 Bonner, J. and Bandurski, R. S., Studies of the physiology, pharmacology and biochemistry of the auxins. Ann. Rev. of Plant Physiol. **3**, 59–86 (1952).

5 Friedericksen, I., Über Funktionen des Mangans im Stoffwechsel der höheren Pflanzen. Planta **34**, 67–87 (1944).

6 Galston, A. W., Bonner, J., and Baker, R. S., Flavoprotein and peroxidase as components of the indole acetic auxin oxidase system of peas. Arch. Biochem. Biophys. **42**, 456–470 (1953).

7 Gustafson, F. G., Influence of external and internal factors on growth hormone in green plants. Plant Physiol. **21**, 49–62 (1946).

8 Hoskins, F. A., Changes in activities of several enzymes during germination and seedling development in corn (*Zea mays* L.). Plant Physiol. **30**, 74–78 (1955).

9 Jones, J. W., Effect of reduced oxygen pressure on rice germination. J. Am. Soc. Agron. **25**, 69–81 (1933).

10 Koneski, M., Proc. Japan Acad. 30: 24–29. 1954. *Cited by* J. L. Liverman, Ann. Rev. of Plant Physiol. **6**, 177–210 (1955).
11 Leopold, A. C., The control of tillering in grasses by auxin. Am. J. Botany **36**, 437–440 (1949).
12 Liverman, J. L., The physiology and biochemistry of flowering. Ph. D. Thesis. Calif. Inst. of Technology, Pasadena, Calif. (1952).
13 Loew, O. and Sawa, S., On the action of manganese compounds in plants. Bull. Coll. Agr. Tokyo Japan **5**, 161–172 (1903).
14 Matsuo, T., Rice Culture in Japan. Yokendo Ltd., Tokyo, Japan (1955).
15 Murato, Y., Osada, A., Iyama, J., and Yamada, N., Photosynthesis of rice plants. IV. Plant factors constituting photosynthetic ability of the rice plants growing on paddy field. Proc. Crop Sci. Soc. Japan. **25**, 133–137 (1957).
16 Olsen, C., Über die Manganaufnahme der Pflanzen. Biochem. Z. **269**, 329–348 (1934).
17 Ordin, L., Applewhite, T. H., and Bonner, J., Auxin induced water uptake by Avena coleoptile sections. Plants Physiol. **31**, 44–53 (1956).
18 Ponnamperuma, F. N., The chemistry of submerged soils in relation to the growth and yield of rice. Ph. D. Thesis. Cornell University (1955).
19 Rao, M. B. V. N., A note on a few experimental observations in Rice Research Sta. Berhampur. Indian J. Agr. Sci. **7**, 733 (1937).
20 Ray, P. M., Destruction of Auxin. *In:* Ann. Rev. Plant Physiol. **9**, 81–118 (1958).
21 Rhind, D. and Thein, V. Ba., Growth and yield studies on irrigated paddy in upper Burma. Indian J. Agr. Sci. **13**, 335–348. (1943).
22 Sekiya, F., Studies on the tillering primordium and tillering bud in rice seedlings. III. The effect of tillering primordium and tillering bud. Proc. Crop. Sci. Soc. Japan. **21**, 10–21 (1952).
23 Skoog, F., Broyer, T. C., and Grossenbacher, K. A., Effects of auxin on rates, periodicity, and cosmotic relations in exudation. Am. J. Botany **25**, 749–759 (1938).
24 Somers, I. I. and Shive, J. W., Iron-manganese relation in plant metabolism. Plant Physiol. **17**, 582–601 (1942).
25 Suzuki, S., On the growth of the rice plant. Agr. Exp. Sta. Formosa Bull. **124**, 1–40 (1917).
26 Toth, S. J., Prince, A. L., Wallace, A. and Mikkelsen, D. S., Rapid quantitative determination of 8 mineral elements in plant tissue by a systematic procedure involving use a flame photometer. Soil Sci. **66**, 459–466 (1948).
27 Tsui, C., The role of zinc in auxin synthesis in the tomato plant. Am. J. Botany **35**, 172–179 (1948).
28 Tsui, C., The effect of zinc on water relations and osmotic pressure of the tomato plant. Am. J. Botany **35**, 309–311 (1948).
29 Tullis, E. C. and Caralley, E. M., Chlorosis of rice induce by iron deficiency. Phytopathology **26**, 111 (1936).
30 Twyman, E. S., The iron-manganese balance and its effect on the growth and development of plants. New Phytologist **45**, 18–24 (1946).

Plant and Soil XIV, no 2 April 1961

STUDY OF SOIL ALGAE

IV. ESTIMATION OF THE NUTRIENT STATUS OF SOIL USING AN ALGAL GROWTH METHOD WITH SPECIAL REFERENCE TO NITROGEN AND PHOSPHORUS

by Y. T. TCHAN, L. N. BALAAM, R. HAWKES* and F. DRAETTE

Department of Agriculture, University of Sydney

INTRODUCTION

The principle and some basic considerations of the method have been discussed in previous papers (Tchan [3 4]). In the present paper further details about the method are given for the estimation of the nutrient status of N and P in soil.

MEDIA

(A) *Medium for N-assay*

The basic medium is that described by Allen and Arnon [1], with minor modifications.

(a) Major elements

0.001 *M* $MgSO_4$	0.004 *M* NaCl
0.0005 *M* $CaCl_2$	0.002 *M* K_2HPO_4

(b) Trace elements

Fe-EDTA complex	4 ppm of Fe	$Na_2B_4O_7.10H_2O$	0.5 ppm of B
$MnCl_2.4H_2O$	0.5 ppm of Mn	NH_4VO_3	0.01 ppm of V
$NaMoO_4.2H_2O$	0.1 ppm of Mo	$NiCl_2.6H_2O$	0.01 ppm of Ni
$ZnSO_4.7H_2O$	0.05 ppm of Zn	$Co(NO_3)_2.6H_2O$	0.01 ppm of Co
$CuSO_4.5H_2O$	0.02 ppm of Cu		

(c) NH_4NO_3 solution. 100 ppm of N

---

* Present address: Royal Alexandra Hospital for Children, Sydney, N.S.W., Australia.

(B) *Medium for P-assay*

(a) Major solution

0.001 *M* $MgSO_4$ — 0.004 *M* NaCl

0.0005 *M* $CaCl_2$ — 0.002 *M* K_2SO_4

(b) The trace elements solution is as above.

(c) KH_2PO_4 solution 100 ppm of P.

METHODS

(a) *pH determination*

The pH of the soils and of the media used for algal growth were determined by means of a glass electrode. The media must have a similar pH to the soil. However, a variation of $\pm$ 0.2 unit is permissible.

(b) *Choice of quantity of soil to be used for assay* (See Discussion).

For P-assay, 1 g of soil is used for 100 cc of medium. For the N-assay, 5 g of soil is used with 50 cc of medium.

(c) *Preparation of complete solution*

The complete solution for the N-assay is obtained by adding sufficient ammonium nitrate solution into (A) (a + b) to make a final concentration of 10 ppm of N. For the P-assay, phosphate solution is added into (B) (a + b) to make a final solution of 10 ppm of P.

(d) *Preparation of assay solution*

The assay solutions do not receive the nutrient element under test. They constitute the minus-N or minus-P assay flasks.

(e) *Preparation of algal suspension*

A mixed culture of algae is passed through a gauze of stainless steel to eliminate large clumps or long filaments of algae. The suspension is then centrifuged and washed by repeated centrifugations (generally 3 washings suffice) with distilled water. The final suspension is diluted until an optical density of 0.045 at 6800 Å is obtained with a cell of 10 mm diameter. This suspension is added to the solution at a rate of 4 cc per liter.

(f) *Procedure*

The appropriate amount of soil is weighed and transferred to chemically clean flasks. The solution (major elements, trace elements, and algal suspension), after having been adjusted to the pH of the soil, is added to all flasks so that inoculum and all chemical constituents are identical in every

flask. NH_4NO_3 or KH_2PO_4 is added to obtain the complete solution. The final volume is obtained by adding the necessary amount of distilled water.

(g) *Incubation*

The flasks are incubated in a constant temperature room at 25°C over a battery of fluorescent lamps (Philips, warm tint) for a set period of time. (See Discussion).

(h) *Chlorophyll extraction*

The final extraction of chlorophyll is carried out according to the method published earlier (Tchan [4]).

SOIL SAMPLES

Soil samples were obtained from an earlier experiment. Their fertility level has been tested and their ecological and chemical characteristics have been published and discussed by Beadle and Tchan [2].

RESULTS

The results are summarised in Table 1, which gives the response of a higher plant (oats) (Beadle and Tchan [2]) and the results obtained with algae. The experimental data are expressed as percentage response using the growth obtained from the complete solution as 100 per cent.

DISCUSSION

(*1*) *Determination of incubation time for the assay*

The results reported below (Table 2) are suitable as a basis for discussion. Two soils were used, Soil A for N and Soil B for P determination. The amounts of chlorophyll obtained with varying duration of incubation are expressed as percentages using the results obtained in the complete solution as 100 per cent.

From these data (p. 150) it may be seen that results become very similar after an incubation period of 8 days. For the two elements under consideration, no appreciable error is expected when the duration is over 10 days. The most convenient time is about 2 weeks. The incubation should not exceed 3 or 4 weeks for N (Tchan [4]).

TABLE 1

Comparison between the algal response and plant response						
Soil	pH	Habitat	Percentage of plant response (oats)		Percentage of algal response	
			P	N	P	N
1	6.9	Rocky ridge	25	26	23.5	33
2	7.3	,,	—	36	—	39
3	7.0	Stable dune	72	16	94	12.5
4	6.3	,,	—	40	—	52
7	8.1	,,	—	50	—	51.5
19	6.2	Rocky ridge	72	—	56	—
20	7.6	,,	67	—	63	—
22	6.0	,,	—	48	—	62
24	7.3	,,	—	56	—	45.5
31	7.1	Stable dune	104	106	98	80
33	7.4	,,	—	65	—	73
40	6.0	,,	—	38	—	46.5
41	5.9	,,	—	43	—	51.5
42	5.4	,,	36	21	28	15
44	6.9	,,	42	15	40	24.5
46	6.0	,,	—	38	—	56.5
48	6.5	,,	81	78	64	60
49	6.0	,,	—	35	—	61.5
50	6.3	,,	56	—	60	—
*	—	,,	—	101	—	98.5

TABLE 2

The influence of duration of incubation on chlorophyll production			
Element assayed	Duration	10 ppm (= 100% of growth)	O ppm
Soil A			
N	8 days	100	55.5%
	9 days	100	60%
	11 days	100	61%
Soil B			
P	4 days	100	50%
	8 days	100	13%
	14 days	100	14%

(2) *Determination of quantity of soil for the assay*

The quantity of soil under assay conditions varies for each element. A soil known to be deficient in P has been used to illustrate this point. The ratios of soil solution are as follows: — 0:100, 1:100, 2:100, 4:100. The results are summarized in Table 3.

TABLE 3

Production of chlorophyll in various soil/solution ratios (only mean value of duplicate is reported here). The production of chlorophyll has been expressed as percentage (10 ppm P in solution = 100% growth) and optical density

P in solution, ppm	Soil/solution ratio (g : cc)							
	0 : 100		1 : 100		2 : 100		4 : 100	
	%	Optical density	%	Optical density	%	Optical density	%	Optical density
0	21.7	0.013	34	0.024	61	0.037	81	0.055
10	100	0.600	100	0.065	100	0.061	100	0.068

When the results are expressed as percentages using the growth in 10 ppm as 100 per cent (percentage column), it is apparent that the response to the addition of P is decreased with increased quantity of soil used. In other words, the more soil that is used, the more chlorophyll is produced in the 0 ppm solution. It can be seen that the percentage of chlorophyll produced is related to the quantity of soil used.

This experiment has an important bearing on the problem of correlation.

The basic argument for the soil fertility bioassay using micro-organisms, is the correlation obtained from such an assay compared to the results given by an assay using higher plants. The validity or the usefulness of the microbiological assay is accepted according to the degree of similarity in results obtained in the two types of assays. Since the results obtained using higher plants (which are regarded as reference) can be variable according to the species used and cultural conditions (Tchan [4]), it becomes difficult to obtain a good correlation between results obtained by a fixed procedure of the algal method and the results of trials from a great number of widely different species of higher plants. This difficulty, which has been overlooked by other workers, required a fresh approach. The results obtained in the above experiment indicate that differences in the amounts of chlorophyll produced by a solution containing P and one not containing P vary according to the quantity of soil used. In the present case, the use of 1 g of soil to 100 cc of solution gives a strong response to the addition of P. One may conclude that the soil is rather deficient in P. If 4 g of soil is used, the response is rather

weak. A contrary conclusion can be drawn. By varying the quantity of soil used, any degree of response may be obtained within reasonable limits. It becomes apparent that the quantity of soil suitable to any particular experimental requirement may easily be chosen. (For each soil group as well as for any particular plant and cultural conditions.) Two methods are therefore suggested:

(a) The simplest is to use a soil of known response from pot trial to a nutrient element for a particular plant under investigation. A series of algal cultures is set up with different quantities of soil for a certain volume of solution similar to the experiment discussed above. The quantity of soil is determined by that giving a result very close to plant response. A practical example will clarify the principle. For example, for Soil No 44 for P and Soil No 7 for N the only variable in the experiment is the quantity of soil used. The results are summarized in Table 4 and expressed as percentages.

TABLE 4

Comparison between the plant growth and chlorophyll production with various quantities of soil for the algal method, using 100 cc of medium per flask. Figures are given in percentages. (Complete solution = 100%)

Plant response		Algal response		
Minus solution	Complete solution	Various quantities of soil	Minus solution	Complete solution
— N			— N	
50	100	2 g	27	100
		10 g	51.5	100
		20 g	77	100
— P			— P	
42	100	0.5 g	22	100
		1 g	40	100
		3 g	90	100

The quantity of soil suitable is 10 g for N-estimations and 1 g for P-estimations with 100 cc of medium. These quantities give results comparable with those obtained by plant test (Oats in the present case). It is valid only for oats and this particular soil.

(b) An alternative solution would be to choose arbitrarily a certain quantity of soil for carrying out the assay. The results obtained are multiplied by an appropriately chosen factor to obtain a response correlated as closely to the plant trial as possible. Such a procedure has definite advantages. It needs only a change of factor

to adapt the method for any crop and any cultural techniques.

However, the technique indicated in (a) can be used as a means of fixing the arbitrary quantity used in (b). In the case of a particular crop in a region of homogeneous soil structure and climatic conditions, method (a) is regarded as more suitable. It is suggested that a combination of the two techniques should be used to obtain the best results.

The above techniques seem to be very artificial. The comparison of algal growth (or the growth of any other micro-organisms) to that of a higher plant can not be regarded as anything but artificial. Therefore, it is justifiable to use such a technique for the purpose of obtaining the best correlation possible.

If the argument is carried too far, one would be soon in a position of having to determine the quantity of soil to be used for every particular plant in any cultural condition. The method would then not be able to serve its original purpose defined in the previous paper (Tchan [4]).

The important point is to assess whether the quantity of soil arbitrarily fixed could be used for a reasonable number of other soils. This point is tested by the statistical analysis of the results obtained.

(3) *Statistical analysis of correlation between responses determined by the algal method and trials with higher plants*

The quantities of soil determined by method (a) with soil No 7 for the N-assay and No 44 for P were used.

The results of pot trials with oats are expressed as 100 per cent of the growth obtained in a complete solution (Beadle and Tchan [2]). The growth in the solution minus the element under test is expressed as a percentage of the complete solution.

The same method of expression is also used for the algal assay. The algal growth in the complete solution is taken as 100 and the algal growth in the solution minus the element is expressed as a percentage.

For this discussion, the P- and N-assays are used. The results are summarized in Table 5.

The statistical analysis shows that the standard error per determination $\times$ 100/mean are of the same order for the oats trial and the algal method. This indicates that the inherent or residual

TABLE 5

Comparison of oats pot trials with the algal growth.
Statistical analysis of figures from Table 1

Statistical characteristic	Series A Response to P, %		Series B Response to N, %	
	Oats	Algae	Oats	Algae
Number in series	9	9	17	17
Average or general mean	61.9	58.5	47.8	50.7
Range	25–104	23.5–98	15–106	12.5–98.5
Standard error per determination	24.5	25.9	26.6	22.5
Coefficient of variation	39.6%	44%	56%	44%
Regression equation	$y = 0.85\,x + 12.2$		$y = 1.04\,x - 5.1$	
Correlation coefficient	$r = 0.90$ **		$r = 0.87$ **	

** Correlation coefficient significant at 1% level.

errors of both methods are approximately the same for P and N.

To provide a means of predicting plant growth using the results obtained by the algal method, regression equations of the form $y = bx + c$, where y is the percentage response as determined by the plant method and x is the percentage response by algal method, were calculated for both series.

The two regression equations are shown in Table 5. To test the significance of the regression equations and the correlations, an analysis of variance was carried out. The results are summarized in Table 6.

The mean squares due to the regression were, for both series, significantly greater than those due to deviations from the regression line indicating that there is a real association between the variables

TABLE 6

Analysis of variance

Source of variation	P Series A			N Series B		
	D. F.	Mean square	F	D. F.	Mean square	F
Regression function	1	3880.71	29.2 **	1	8613.24	47.1 **
Deviation from regression line	7	132.88		15	182.92	
S. E. per estimate		11.53			13.52	
% of general mean		18.6			28.3	

** = F value significant at 1% level.

of the type indicated by the regression equations used above. Therefore, the correlation coefficients, ($r = 0.90$ and $r = 0.87$) are highly significant.

Before using the regression equations:

$$y = 0.85\,x + 12.2 \text{ (Series A)}$$
$$y = 1.04\,x - 5.1 \text{ (Series B)}$$

to predict plant response by the algal method it must be ascertained whether the danger associated with extrapolation has not been overlooked. In this case, both series cover a wide range of responses (23.5 to 98.0 per cent for phosphorus: 12.5 to 98.5 per cent for nitrogen) and a great variety of ecological and soil conditions. Thus the danger mentioned above is not likely to be important.

Further, whilst the independent variate, percentage response as determined by algal method is subject to error, it was stated by Yates [5] that "Errors in the independent variates do not effect the validity of the ordinary tests of significance, provided that only differences from zero are being tested, and the coefficient as ordinarily evaluated will always be the best coefficients for the purpose of prediction from subsequent observations subject to similar errors."

A summary of the responses as estimated from the regression equations is given in Table 7.

The results give the estimated plant growth according to the regression equations based on the algal results arbitrarily chosen.

TABLE 7

Correlation of plant and algal growth			
Series A, P-assay		Series B, N-assay	
Algal growth as percentage	Plant growth estimated by regression equation	Algal growth as percentage	Plant growth estimated by regression equation
10	21	10	5
20	29	20	16
30	38	30	26
40	46	40	37
50	55	50	47
60	63	60	57
70	72	70	68
80	80	80	78
90	89	90	89

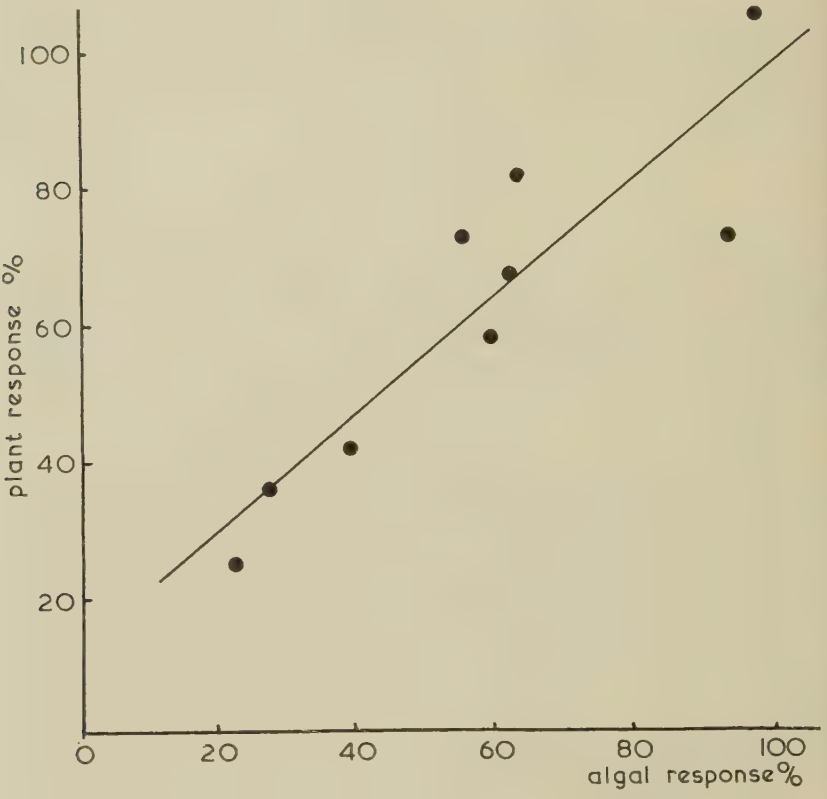


Fig. 1. Correlation of plant and algal growth (P) with regression line.

Figures 1 and 2 are prepared using the experimental data points (percentage of response by higher plants against the percentage of response by algae). These lie close to the regression lines which were obtained by statistical estimation. The overall picture suggests that the regression equations could be used with advantage for the prediction of higher-plant response based on results obtained by the algal method.

CONCLUSION

From the graphs and the general statistical considerations under the experimental conditions the following conclusions can be stated.

The inherent errors of the algal method and oats pot trials for P and N are of the same magnitude.

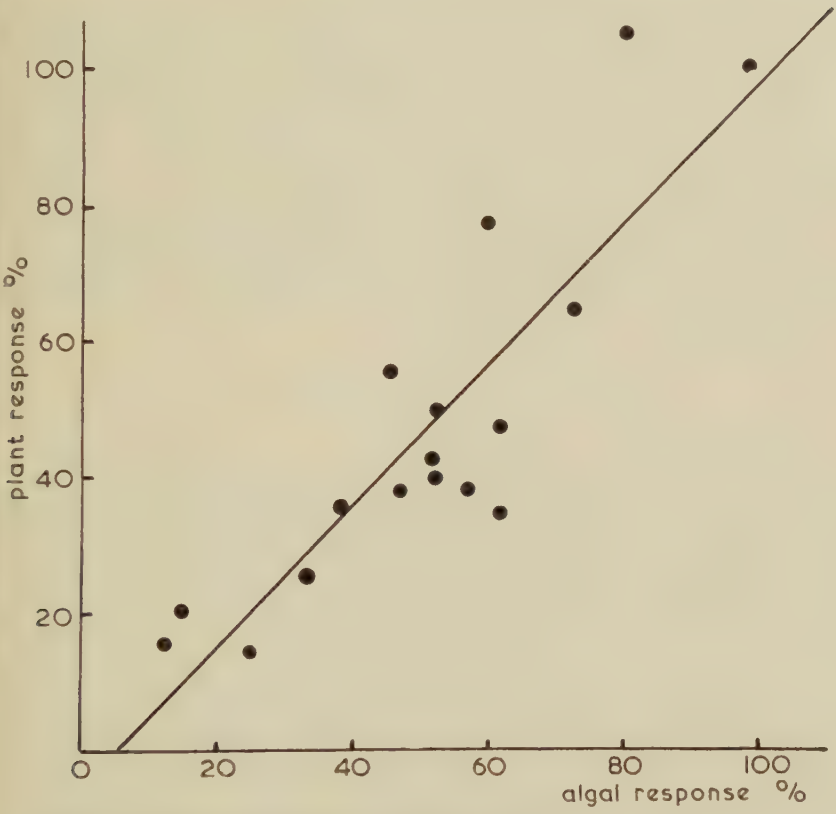


Fig. 2. Correlation of plant and algal growth (N) with regression line.

The correlations between the oats pot trial and algal method for N and P obtained under the experimental conditions are highly significant. Therefore, the regression equations could with safety be used for prediction of plant response from data obtained with the algal method.

On statistical grounds, the algal method would be able to replace the pot trials provided the conditions are similar to those of the present experiments.

The high degree of correlation indicated by the statistical analysis provides a sound basis for the use of the algal method. In practice, a much smaller correlation could be regarded as adequate for recommending the use of the algal method for agronomic purposes (*e.g.* fertilizer recommendations, *etc.*). By modifying slightly one

could adapt the method for a variety of uses for which the pot trial might not be suitable. Since the present study is based on a relatively limited number of soils it would be most desirable that more experimental data should be obtained and a more detailed statistical analysis be carried out. The difficulty of interpreting the present results for field use is the same as that for pot trials.

The principle of using different quantities of soil for the assays is pertinent and its application need not be limited to the algal method. It is very easy to extend this principle to many other fields where the bioassay is in use.

SUMMARY

For practical purposes it is likely that the algal method for bioassay of soil fertility could be used in agriculture. It has the advantage of being easier to handle and more adaptive than pot trials. In this paper only P and N have been discussed. The evidence from our unpublished work indicates that the method could be extended to other elements and more general applications.

ACKNOWLEDGEMENTS

The writers are indebted to Dr. J. S. Bunt and Associate Professor N. Collis-George of the Faculty of Agriculture, University of Sydney, for reading the manuscript and for helpful criticism; also to Miss. R. A. Webb, Laboratory Assistant, for help with the work.

Financial assistance by the Commonwealth Research Grants Fund of the University of Sydney and the Wheat Industry Research Council (Commonwealth) are likewise gratefully acknowledged.

Received March 22, 1960

REFERENCES

1 Allen, M. B. and Arnon, D. I., Studies on nitrogen-fixing blue-green algae. I. Growth and N-fixation by *Anabaena cylindrica* (Lemm.). Plant Physiol. **30**, 366–372 (1955).

2 Beadle, N. C. W. and Tchan, Y. T., Nitrogen economy in semi-arid plant communities. Part I. The environment and general considerations. Proc. Linnean Soc. N. S. Wales **80**, 62–70 (1955).

3 Tchan, Y. T., Estimation of nutrient elements in soil by algae. Principle of the method. Congr. Intern. Sci. Sol 6me Congr. Paris **C**, 249–251 (1956).

4 Tchan, Y. T., Study of soil algae. III. Bioassay of soil fertility by algae. Plant and Soil **10**, 220–232 (1959).

5 Yates, F., Tests of significance of the differences between regression coefficients derived from two sets of correlated variates. Proc. Roy. Soc. Edinburgh **59**, 184–194 (1939).

Plant and Soil XIV, no. 2 April 1961

MOLYBDENUM AND THE FIXATION OF NITROGEN IN *CASUARINA* AND *ALNUS* ROOT NODULES

by E. J. HEWITT

Long Ashton Research Station, University of Bristol

and G. BOND

Botany Department, University of Glasgow

Evidence that there is a specific requirement for molybdenum in the nitrogen fixation proceeding within legume nodules was reviewed by Anderson [1]. In brief, it has been shown that the concentration of molybdenum in the nodule tissues is usually substantially greater than in other parts of the legume, and that, under conditions in which the plant is wholly or mainly dependent on the fixation of elemental nitrogen for its growth, an inadequate supply of molybdenum results in the appearance of symptoms of nitrogen deficiency in the plant, evidently because of a reduction in fixation, though there is no diminution in the number of nodules formed. (It is of interest that Mulder *et al.* [15] have recently shown that fixation of nitrogen in nodulated legumes previously deprived of molybdenum is stimulated in a matter of hours after the molybdenum supply is restored). Further, there is evidence that legumes utilising elemental nitrogen have a higher molybdenum requirement than those using combined nitrogen. As indicated by the studies reviewed by Hewitt [9], the symptoms induced by molybdenum deficiency in legumes grown with combined nitrogen are not those of nitrogen deficiency but resemble those shown by plants in general when deprived of molybdenum [8], these being possibly attributable to the excessive accumulation of unreduced nitrate demonstrated in the tissues.

Thus the evidence is wholly consistent with the fulfilment by

molybdenum in the legume nodule of some specific role in the fixation, as has been established with certainty for fixation in free-living organisms [20]. Now that it has been shown [3 4] that there is a substantial further group of Angiosperms bearing nitrogen-fixing root nodules which are tenanted by organisms quite different in nature from the rhizobia of legume nodules, it becomes of interest to enquire whether a special importance for molybdenum can be demonstrated in these additional instances of biological nitrogen fixation.

METHODS

Plant material

Two examples of nodule-bearing non-legumes were examined in this study, namely *Casuarina cunninghamiana* (sub-tropical) and *Alnus glutinosa* (cold-temperate). Their small seed weight (of the order of 1 mg) was one consideration influencing the selection of these species for initial investigation. In previous studies by one of us (G.B.) and other collaborators, large numbers of nodulated plants of these species have been grown, and by virtue of the fixation of nitrogen occurring in the nodules, the plants have consistently shown vigorous development in a rooting medium devoid of combined nitrogen; molybdenum was supplied to the plants on these previous occasions, in view of its known importance for other nitrogen fixers. In the present studies the effect of molybdenum deprival has been investigated, both for nodulated plants growing without access to external combined nitrogen and for non-nodulated plants supplied with nitrate nitrogen.

Cultural methods

A water-culture technique previously described for work with these plants [2 6] was used, with modifications to minimise contamination with molybdenum. The plants were grown by daylight in the greenhouses attached to the Botany Department, University of Glasgow. Seed was sown in purified sand [7], and seedlings were transplanted into water culture when the epicotyl was about 0.5 cm high (Casuarina) or when one true leaf had emerged (Alnus).

Containers for the plant cultures consisted of 800-ml Pyrex beakers which after normal cleansing were 'steamed' from boiling HCl (1 : 4, v/v) for thirty minutes and then rinsed with re-distilled water (see below). The beakers, provided with black paper wrappings, were covered by black polythene squares bored with five holes for the plants; the squares, after normal cleansing, were immersed for three hours in HCl (1 : 1, v/v) at 60 to 70°C, followed by rinsing. Thin sections of rubber tubing were placed resting over the holes in the polythene squares, the roots of the seedlings

being inserted through these sections and the plants supported at a suitable level by means of small rubber wedges; these items were cleansed by acid-treatment or by repeated simmering in culture solution. The photographs provided will supplement this description of the cultural arrangement.

Water was prepared by re-distilling from Pyrex glass stills that collected from a metal still. The basal culture solution contained $CaCl_2$, K_2SO_4, $MgSO_4$, Na_2HPO_4, NaH_2PO_4, and iron versenate (Fe–EDTA), and was prepared from reagents purified from molybdenum at the Long Ashton Research Station by treatment with copper sulphate and hydrogen sulphide [7 10 18]. The iron versenate was prepared as described by Jacobsen [11] from ferrous sulphate and sodium versenate which were purified separately by copper sulphide co-precipitation before mixing. The concentrations (in ppm) supplied by this solution were as follows: Ca 48, K 55, Mg 12, P as PO_4 16, S as SO_4 38. A micronutrient and other trace-element supplement (not including molybdenum) was added in the form of 1 ml of a Hoagland A–Z solution (as modified by Templeman [19]) per litre of culture solution. Where desired, sodium nitrate (molybdenum-free stock) providing 56 ppm of nitrogen was furnished to the plants. Certain cultures received 0.05 ppm of molybdenum as sodium molybdate, a level which has been found satisfactory with varied plant species [8]. The pH of the freshly prepared solution was 6.5. The culture solution in the beakers was renewed fortnightly in early stages of growth and more frequently later.

In order to ascertain in advance whether the above technique was in fact giving satisfactory control over the access of the plants to molybdenum, three separate tests were made with tomato plants during the investigation. In each test, tomato plants which had not received any deliberate addition of molybdenum showed typical molybdenum-deficiency symptoms [8] after some twelve days' growth in water culture with nitrate nitrogen; the addition of molybdenum at the level indicated above resulted in a rapid recovery of the plants.

Inoculation

Since cultures of the endophytes of these nodules were not available, the formation of nodules was induced, where desired, by the application to the root system of an inoculum prepared from nodules of older plants of the same species. The nodules were procured from greenhouse plants in the case of Casuarina and from field plants in that of Alnus. A quantity of washed nodule material (10 g in Casuarina, 5 g in Alnus) was ground in 100 ml of water with a little purified sand added, and after filtration through muslin one drop of the inoculum so prepared was applied to the root system of each plant. For Alnus use was also made of two weaker inocula prepared by dilution of a portion of the original inoculum to one fifth and one twenty-fifth respectively; these inocula are subsequently designated *A*, *B* and *C* in order of decreasing strength. In Alnus inoculation was effected soon after the seedlings had been transferred to water culture, but in Casuarina in view of previous experience inoculation was delayed

until the plants had been cultured in the presence of nitrate nitrogen (without any addition of molybdenum) for one month subsequent to transplanting.

The application of the above inocula was obviously likely to introduce some molybdenum into the cultures. The molybdenum contents of the actual inocula were not determined, but analyses of nodules similar to those used in preparing the inocula indicated that they were very small *. This introduction of molybdenum would obviously be minimised by using the weakest inoculum that still produced adequate nodulation, hence the employment of different strengths in the Alnus experiments. In order to provide a corresponding category of non-nodulated plants fed with nitrate, autoclaved inoculum was added in the above amount to certain beakers containing plants of the latter type, the same interval (12 days) being allowed before the first renewal of the culture solution.

Harvesting

In the species under investigation, plants supplied with combined nitrogen grow much more rapidly in the early stage than those dependent on nodule nitrogen, owing to the time required for the nodules to form and to become functional. In view of this the plan formulated was to harvest the nitrate plants when they were becoming too large for the containers employed, but to continue the nodulated plants in growth until those supplied with molybdenum had attained approximately the same size as that at which the corresponding nitrate plants had been harvested. The respective significance of molybdenum in the growth of nitrate and nodulated plants could then be assessed by comparison of the effects of molybdenum deprival.

At harvest, dry weights were determined by heating the plant material in Pyrex containers to constant weight at 95°C, and total nitrogen estimated by the Kjeldahl process. For nitrate nitrogen steam distillation as 2:4-dinitroxylen-5-ol as described by Sørenson [17] was used, while molybdenum was determined by the dithiol method of Piper and Beckwith [16] modified at Long Ashton to accommodate a lower limit of about 0.02 μg molybdenum.

DATA OBTAINED

a. CASUARINA

Non-nodulated plants supplied with nitrate nitrogen

Seedlings were transplanted into water culture on 24th May, 1958, three treatments being included, namely plants supplied

* More recently the analysis of an Alnus inoculum (strength *A*) prepared in another connection showed a molybdenum content of 0.05 ppm.

with 0.05 ppm of molybdenum, those not supplied with that element, and those given a single application of autoclaved inoculum.

After some two months' growth the plants not supplied with molybdenum developed a chlorosis in the younger regions of the lateral branches of the shoot, and in some plants in the leading shoot also. After a further interval these same regions withered. The experiment was continued until 25th September, 1958, when all plants were harvested. The plants supplied continuously with molybdenum had continued to grow strongly, while those denied that element had become increasingly crippled, as indicated in Plate 2A; the symptoms shown — chlorosis followed by dying-back of the younger parts of the branches, without anthocyanin formation — were not those typical of nitrogen deficiency.

The plants given the single application of autoclaved inoculum remained healthy and indistinguishable from the plus-molybdenum series, except that towards the end of the growth period some of the plants showed a slight tendency to chlorosis and withering in a few lower branches. These plants are considered again below.

Harvest data are presented in Table 1A. They confirm the inferior growth of the plants denied molybdenum as compared with that of the plants given a constant supply; percentage nitrogen was slightly higher in the former plants, a further difference being that nitrate was over five times more abundant in the tissues.

TABLE 1A

Harvest data for non-nodulated Casuarina plants grown for 18 weeks with nitrate nitrogen supplied *

Treatment	No. of plants	Mean height of shoot (cm) **	Mean dry weight per plant (g) **	Percentage total N in dry matter	Total N per plant (mg)	Percentage nitrate in dry matter
(1) Molybdenum added	20	30.0	0.484	3.35	16.2	0.03
(2) Molybdenum not added	30	19.5	0.254	3.67	9.3	0.17
(3) Autoclaved inoculum added .	10	29.3	0.478	—	—	—

* The molybdenum contents of these plants are given in Table 1B.

** In respect of height of shoot and dry weight per plant the differences between means have he following significance at $P = 0.05$ and 0.01: Treatments (1) and (3) > Treatment (2), reatment (1) = (3).

TABLE 1B

Molybdenum contents of plants of Table 1A					
Treatment	Mo ppm in dry matter		Mo per plant (μg)		
	Shoot	Roots	Shoot	Roots	Total
Molybdenum added *	5.30	28.9	2.0	2.9	4.9
Molybdenum not added	0.08	0.17	0.02	0.01	0.03
Autoclaved inoculum added . . .	1.45	2.03	0.56	0.18	0.74

* Molybdenum supplied during the growth period amounted to 80 μg per plant.

The data provided in Table 1B show that the molybdenum content of the plants not deliberately supplied with that element was satisfactorily low. Substantial amounts of molybdenum accumulated in the tissues of plants supplied with the element, especially in the roots. The molybdenum content of the plants given autoclaved inoculum was greater than could be explained on the basis of that likely to have been included in the inoculum, a finding which may have its origin in a suspected error in the application of nutrients at an early stage in the experiment. These plants thus fail to serve their intended purpose, namely to provide for a comparison between nitrate plants and nodulated plants both supplied only with the inoculum molybdenum. They do show, in keeping with the findings of previous workers for other plants, that a relatively low level of molybdenum in the tissues suffices for the growth of Casuarina supplied with nitrate.

Nodulated plants dependent on fixation of elemental nitrogen

Seedlings for this part of the investigation were also transplanted on 24th May, 1958, and were inoculated on 19th June. Practically all plants were nodulated by early August, but owing to the time taken for the nodules to become functional the growth made by the plants during this first season was small. By the end of October the height of the shoots was only of the order of 10 cm, and there was no perceptible difference between the molybdenum treatments.

The plants were retained over the following winter, and differences soon became apparent between the two treatments when growth was resumed in April, 1959. The plants receiving molybdenum became increasingly vigorous in growth, and it was obvious that the nodules were providing sufficient fixed nitrogen to permit

healthy plant development in a rooting medium free of combined nitrogen. The plants denied access to molybdenum were notably slower in growth and developed chlorosis in the lower regions of the branches of the shoot; red pigmentation appeared in the same regions, together with a darkening of the roots. The plants were harvested on 15th July, 1959. Plate 1A indicates the appearance of typical plants at this time, while the data obtained are presented in Tables 2A and 2B.

The data in Table 2A show that the mean height of the shoot in plants given molybdenum was nearly twice that in plants without molybdenum, while the mean dry weight was over three times greater. The percentage-total-nitrogen figures confirm that the minus-molybdenum plants were deficient in nitrogen, as suggested by the visual symptoms described above. The actual fixation of elemental nitrogen is obtained by deducting from the mean total nitrogen per plant the amount 0.6 mg, this being the mean nitrogen content of five non-nodulated plants grown in the solution free of combined nitrogen (except during the initial period of approximately one month when all the plants received nitrate). Thus mean fixation in the presence of molybdenum was 19.1 mg per plant, but in the absence of that element was only 3.1 mg.

The data presented in Table 2B show that despite the long duration of the experiment the control over the access of the plants to molybdenum remained satisfactory, since the molybdenum content of the plants not deliberately given that element was very low. The relatively high level of molybdenum in the nodules

TABLE 2A

Harvest data for nodulated Casuarina plants grown for 14 months in solution free of combined nitrogen *

Treatment	No. of plants	Mean height of shoot (cm) **	Mean dry weight (g) **		Percentage total N in whole plant dry matter	Mean total N per plant (mg)
			Nodules	Whole plant		
Molybdenum added . .	17	29.8	0.065	1.000	1.97	19.7
Molybdenum not added	22	16.6	0.033	0.292	1.27	3.7

* The molybdenum contents of these plants are given in Table 2B.

** The means are significantly different at $P = 0.01$ in respect of height of shoot and whole plant dry weight, and at $P = 0.05$ in respect of nodule dry weight.

TABLE 2B

Molybdenum contents of plants of Table 2A							
Treatment	Mo ppm in dry matter			Mo per plant (μg)			
	Nodules	Shoot	Roots	Nodules	Shoot	Roots	Total
Molybdenum added * .	90.9	47.3	46.9	5.9	35.6	8.5	50.0
Molybdenum not added	0.61	0.11	0.05	0.02	0.02	0.004	0.044

* Molybdenum supplied during the growth period amounted to 240 μg per plant.

is noteworthy, and it is particularly striking that in the molybdenum-deficient plants some 45 per cent of the total molybdenum was contained in the nodules, indicating a very high accumulative capacity.

On the question of the relative importance of molybdenum for nitrate plants and nodulated plants these data for Casuarina are less informative than intended, chiefly because the desirable condition that the plus-molybdenum plants of each category should be of approximately similar size and dry weight at harvest was not realised. Molybdenum is clearly necessary for both types of plant, but the evidence does not allow it to be said that the effects of deprival are more drastic with one type than with the other.

b. ALNUS

Non-nodulated plants supplied with nitrate nitrogen

Seedlings were transplanted four per beaker into water culture on 30th June, 1959, four treatments being included, namely with and without molybdenum, and with autoclaved inocula *B* and *C* (see p. 161). All plants showed rapid growth. For the first six weeks there was no clear difference between the various treatments, but during the remaining two weeks of the growth period allowed the leaves of the plants not supplied with molybdenum acquired a slightly paler shade of green, of which there is some indication in Plate 1B. The same tendency was shown in smaller degree by the plants which had received autoclaved inoculum.

Data are provided in Tables 3A and 3B. As indicated in the statistical note appended to Table 3A, there was no significant difference in height of shoot between the treatments, but the

TABLE 3A

Harvest data for non-nodulated Alnus plants grown for 8 weeks with nitrate nitrogen supplied *

Treatment	No. of plants	Mean height of shoot (cm) †	Mean dry weight per plant (g) †	Percentage total N in dry matter	Total N per plant (mg)	Percentage nitrate in dry matter
(1) Molybdenum added	8	18.8	0.554	3.14	17.4	0.324
(2) Molybdenum not added	8	17.0	0.451	2.25	10.1	0.515
(3) Autoclaved inoculum added **	16	17.0	0.454	2.64	12.0	0.516

* The molybdenum contents of these plants are given in Table 3B.

** The data for plants receiving *B* and *C* inoculum have been combined since the plants were indistinguishable.

† In respect of height of shoot the means are not significantly different at $P = 0.05$. The same applies to dry weight at $P = 0.01$, but at $P = 0.05$ dry weight for Treatment (1) is significantly greater than those for Treatments (2) and (3).

TABLE 3B

Molybdenum contents of plants of Table 3A

Treatment	Mo ppm in dry matter		Mo per plant (μg)		
	Shoot	Roots	Shoot	Roots	Total
Molybdenum added * . . .	43.5	69.4	18.5	9.0	27.5
Molybdenum not added . .	0.19	0.71	0.06	0.08	0.14
Autoclaved inoculum added	0.11	0.32	0.04	0.04	0.08

* Molybdenum added during the growth period amounted to 60 μg per plant.

difference in mean dry weight between the plants regularly supplied with molybdenum and the two other categories was significant at $P = 0.05$ though not at $P = 0.01$. It is clear that there was some derangement of metabolism in the absence of molybdenum; thus percentage and absolute total nitrogen were reduced, indicating that nitrate uptake had been depressed. On the other hand percentage nitrate was substantially increased in the absence of molybdenum, and separate analyses of root and shoot showed that this nitrate accumulation was occurring chiefly in the root tissues, where 23 per cent of the total nitrogen was in the form of nitrate, the corresponding figure for the plants given molybdenum being 11 per cent.

The molybdenum data in Table 3B show that when that element was provided, these Alnus plants accumulated much higher levels and a much higher proportion of that provided than did the corresponding plants of Casuarina (Table 1B). The molybdenum contents of the plants not deliberately supplied with the element, though satisfactorily low, appear slightly to exceed those of plants given autoclaved inoculum; this is not in accord with the more pronounced visual symptoms shown by the plants of the former category and the evidence of somewhat greater interference with nitrate uptake.

Nodulated plants dependent on fixation of elemental nitrogen

In a first, small-scale experiment, seedlings were transferred to water culture on 2nd May, 1959, and inoculated three days later. A single addition of 4 mg combined nitrogen per beaker of five plants was given to assist the plants over the period of nodulation. A distinct difference between the two treatments first became obvious some nine weeks from inoculation, the plants without molybdenum being now paler and less vigorous. The difference in growth increased in degree as the plants receiving molybdenum continued to grow strongly, and was very marked by harvest on 6th August, the deficient plants now showing severe chlorosis. Typical plants are illustrated in Plate 1C, while harvest and molybdenum data are presented in the upper parts of Tables 4A and 4B.

The data in Table 4A confirm the great superiority of the plants given molybdenum; the percentage-nitrogen figures confirm the visual impression that the plants deprived of molybdenum were deficient in nitrogen. Fixation is calculated by subtracting 0.6 mg (the mean nitrogen content of non-nodulated plants grown in the culture solution free of combined nitrogen) from the appropriate total-nitrogen figures in Table 4A. Fixation was thus 27.8 mg per plant with molybdenum supplied, but only 2.8 mg in the absence of that element.

The relevant data in Table 4B show a very high accumulation of molybdenum in the nodule dry matter, and quite high levels in other parts of the plant. Molybdenum levels in the plants not deliberately supplied with the element are satisfactorily low; here again the nodules show the highest level.

A second experiment with nodulated plants was started con-

TABLE 4A

Harvest data for nodulated Alnus plants grown for 14 weeks in solution free of combined nitrogen *

Treatment	No. of plants	Mean height of shoot (cm) **	Mean dry weight (g) **		Percentage total N in whole plant dry matter	Mean total N per plant (mg)
			Nodules	Whole plant		
First experiment						
Molybdenum added . .	10	19.6	0.071	1.067	2.66	28.4
Molybdenum not added	6	8.3	0.030	0.284	1.18	3.4
Second experiment						
Molybdenum added . .	20	10.1	0.033	0.511	2.21	11.3
Molybdenum not added	20	7.7	0.023	0.226	1.59	3.6

* The molybdenum contents of these plants are given in Table 4B.

** In both experiments the respective means for height of shoot, dry weight of nodules and dry weight of whole plant are significantly different at $P = 0.01$.

TABLE 4B

Molybdenum contents of plants of Table 4A

Treatment	Mo ppm in dry matter			Mo per plant (μg)			
	Nodules	Shoot	Roots	Nodules	Shoot	Roots	Total
First experiment							
Molybdenum added * .	246.1	79.8	97.5	17.5	65.5	17.1	100.1
Molybdenum not added	2.73	0.66	1.48	0.08	0.12	0.10	0.30
Second experiment							
Molybdenum added * .	217.0	79.7	94.3	7.16	29.2	10.5	46.9
Molybdenum not added	1.85	0.41	0.86	0.04	0.06	0.05	0.15

* Molybdenum supplied during the growth period amounted to 180 μg (First experiment) and 96 μg (Second experiment) per plant.

currently with that involving nitrate plants described in the previous section. Thus seedlings were transplanted into water culture on 30th June, 1959, and inoculated the following day. For reasons already given, the plants grew more slowly than the nitrate plants, necessitating a longer growth period, while owing to the

seasonal shortening in daylength and fall in light intensity they grew less rapidly than the nodulated plants of the first experiment. But again after nine weeks' growth the plants denied molybdenum were smaller and paler in colour, features which were more pronounced at harvest on 8th October. Plate 2B shows typical plants, while data are included in Tables 4A and 4B. In plants supplied with molybdenum the dry weight was more than twice as great and the percentage nitrogen higher than in plants without molybdenum. Fixation per plant, calculated by deducting the control figure (in this instance 0.4 mg) from the total-nitrogen figures, was 10.9 mg compared with 3.2 mg in the plants denied molybdenum. The molybdenum data included in Table 4B resemble those for the first experiment.

In this experiment inocula of types *B* and *C* were employed, and although in the tables the data have been pooled, the original data showed that the plants treated with inoculum *C* were slightly inferior to corresponding plants receiving the *B* inoculum, though only where molybdenum was supplied did the difference attain significance. It was clear that the very weak inoculum *C* had induced poorer nodulation. The molybdenum contents of plants not deliberately supplied with the element did not differ appreciably as between plants treated with *B* and *C* inocula respectively; obviously the molybdenum gained from the inocula was small compared with that acquired from uncontrolled sources, though this also was very small.

The results of this second experiment with nodulated Alnus plants may be compared with those derived from the experiment with nitrate plants already described, since the two experiments were started concurrently and the desired condition of similarity in dry weight between the respective plus-molybdenum plants at harvest was realised. With the nodulated plants, in the absence of molybdenum dry weight fell to less than half, but with the nitrate plants the reduction was only to about four-fifths; visual symptoms also were much less pronounced in the latter plants, particularly in those receiving autoclaved inoculum.

Plate 1

A. Nodulated plants of *Casuarina cunninghamiana* after 14 months' growth in water culture without combined nitrogen. 0.05 ppm of molybdenum was added to the solution in which the plants on the right were grown, but was omitted for those on the left. ($\times \frac{1}{4}$).

B. Non-nodulated plants of *Alnus glutinosa* after 8 weeks' growth in water culture with nitrate supplied. 0.05 ppm of molybdenum was supplied to the plants on the left, but was omitted for those in the centre. The plants on the right received autoclaved inoculum. ($\times \frac{1}{6}$).

C. Nodulated plants of *A. glutinosa* after 14 weeks' growth in water culture without combined nitrogen. 0.05 ppm of molybdenum was supplied to the plants on the right, but was omitted from those on the left. From first experiment. ($\times \frac{1}{5}$).

A

B

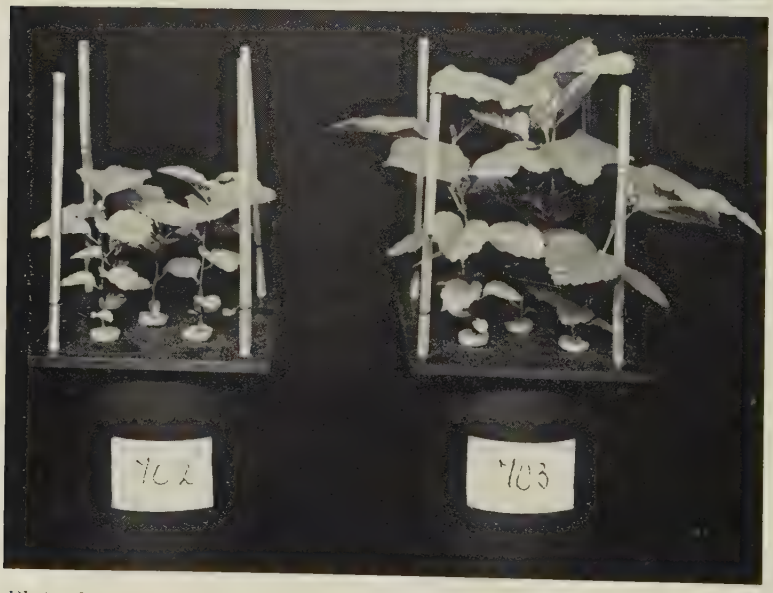


Plate 2

A. Non-nodulated plants of *Casuarina cunninghamiana* after 18 weeks' growth in water culture with nitrate supplied. 0.05 ppm of molybdenum was supplied to the plants on the left, but was omitted for those on the right. ($\times \frac{1}{6}$).

B. As in 1C, except that these are plants of second experiment. ($\times \frac{1}{4}$).

DISCUSSION

It is obvious from the results presented that in Casuarina and Alnus, molybdenum is essential for the growth both of non-nodulated plants supplied with nitrate nitrogen and of nodulated plants dependent on nitrogen fixation. As noted, the evidence available for Alnus shows that a deficiency of molybdenum exercises a more drastic effect on the growth of plants of the latter category than of the former, and though the data for Casuarina are less informative on this aspect it is at least clear that a very small amount of molybdenum suffices for healthy growth of nitrate plants of this genus.

The data indicate that in non-nodulated plants utilising nitrate nitrogen, in both genera, the molybdenum functions in connection with nitrate reduction, as other work has shown to be true of plants in general. Unreduced nitrate accumulates in the tissues of molybdenum-deprived plants. The effect of depriving a nodulated plant of molybdenum, in either genus, is that the capacity for nitrogen fixation is severely impaired. This cannot be attributed to a lack of nodules, for though the absolute weight of nodules per plant was considerably reduced in the absence of molybdenum, relative to plant size the plants were actually better equipped with nodules than were the plants supplied with molybdenum. Thus when mean dry weight of nodules is expressed as a percentage of total plant dry weight, the following values are obtained, that for plants given molybdenum being shown first in each case: Casuarina, 6.5 and 11.3 per cent; Alnus, first experiment 6.7 and 10.6 per cent, second experiment 6.5 and 10.2 per cent. Such data as are available for legumes [9] suggest that a similar effect obtains there. If, in the present experiments, the depression of fixation occasioned by molybdenum deprival were due simply to a reduction in the amount of nodule tissue, such nodules as were formed would have been expected to contribute as much, relatively, to the growth of the plants as those present on plants supplied with molybdenum. Actually they contributed much less, as the above figures show.

Since the main, or possibly the only, role of the nodules is to supply fixed nitrogen to the plant, it must be concluded that in molybdenum-starved plants the nodules are of reduced efficiency in fixation. A measure of this efficiency is obtained when milligrams

TABLE 5

Efficiency of nodule tissue in fixation of nitrogen				
Experiment	Treatment	Mean dry weight of nodules per plant (g)	Mean fixation per plant (mg)	Milligrams N fixed per g of nodule dry matter formed
Casuarina	+ Mo	0.065	19.1	294
	− Mo	0.033	3.1	94
Alnus 1st expt.	+ Mo	0.071	27.8	392
	− Mo	0.030	2.8	93
2nd expt.	+ Mo	0.033	10.9	330
	− Mo	0.023	3.2	140

nitrogen fixed per gram nodule dry matter formed is calculated. A re-statement of the necessary original data and a note of the results of such calculations are provided in Table 5. It will be observed that when molybdenum was not supplied, the efficiency of the nodule tissues in fixation fell substantially.

An obvious explanation of this reduced efficiency would be that molybdenum plays some direct role in the nodular fixation. Alternatively the relation might be indirect and might arise from an improved supply of metabolites to the nodules, or from a more prompt utilisation of nitrogenous products reaching the plant from the nodules, due to some effect of molybdenum exerted in the root or shoot tissues. It is, however, difficult to see what precise effect of this type could be exerted. Nitrate reduction cannot be a process of much significance in a plant dependent on nodule nitrogen, except possibly in the nodules themselves, where nitrate reductase may conceivably be involved in the fixation process as has been suggested for legumes [5]. The isotopic studies of Leaf, Gardner, and Bond [13 14] show that in the non-legume nodules so far examined (including Alnus) the fixed nitrogen, as in those of legume type, accumulates in the nodules in the form of amino acids and amides, and it cannot be doubted that the fixed nitrogen passes from the nodules to the rest of the plant in such reduced forms. It is clearly much simpler and more reasonable to conclude that the beneficial effect of molybdenum is exerted directly on the fixation process in the nodules. The evidence of the greater importance of molybdenum to plants dependent on fixation, at least in Alnus, is consistent with the fulfilment of such a distinctive function.

Also in keeping with this conclusion is the notable accumulation of molybdenum in the nodules of the plants of these experiments, rising to 246 ppm in the dry matter of Alnus nodules; this parallels the findings of Jensen [12] and other authors [9] for legumes. An obvious possible explanation would be that in the nodule (presumably in the endophyte itself) the molybdenum becomes bound into some indiffusible form such as molybdo-protein, for which a catalytic role has been postulated in other instances of nitrogen fixation.

The distribution of molybdenum in the various parts of the nodulated plants presents some features of interest (Table 6). When plants were deprived of molybdenum the nodule tissues or the endophyte, or both together, showed a preferential accumulation of that element over the other tissues, but when adequate levels were provided increasing quantities were translocated to the shoots. This is shown by the change in distribution of molybdenum produced in plants deprived of that element, where percentage uptake increased in the nodules at the expense mainly of the shoots. It may also be noted that the proportion of the molybdenum in the nodules was greater in Alnus than in Casuarina when the element was provided, but that the position was reversed when molybdenum was omitted.

The molybdenum tables in the previous section show that the element was more abundant in root and shoot tissues of nodulated plants than in the corresponding parts of nitrate plants. The reason for this is probably that the experiments with nodulated plants were of greater duration than those with nitrate plants, so that there was greater opportunity for uptake of molybdenum.

TABLE 6

Distribution of molybdenum in nodulated plants

Experiment	Treatment	Percentage of total Mo present in plants at harvest		
		Nodules	Roots	Shoot
Casuarina	+ Mo	11.8	17.0	71.2
	− Mo	45.5	9.0	45.5
Alnus 1st expt.	+ Mo	17.5	17.1	65.5
	− Mo	26.7	33.3	40.0
2nd expt.	+ Mo	15.3	22.4	62.3
	− Mo	26.7	33.3	40.0

When uptake of molybdenum per gram of root and shoot per week is calculated, figures of the same order of magnitude are obtained for nodulated and nitrate plants.

Of the total molybdenum supplied to the plants, the proportions recovered in the dry matter were 21 and 6 per cent in Casuarina for nodulated and nitrate plants respectively. In Alnus the proportions were 56 and 49 per cent for nodulated plants and 46 per cent for nitrate-grown plants. The more rapid uptake by Alnus is doubtless related to the faster growth under the prevailing conditions.

In conclusion it is clear that the relation towards molybdenum in these non-legume, nodule-bearing plants is very similar to that previously demonstrated for legumes; this adds to the many points of resemblance already established [3 4 13 14]. Further, the finding that molybdenum is almost certainly required for nitrogen fixation by the endophytes of the root nodules of Casuarina and Alnus is in conformity with the position demonstrated for other nitrogen-fixing organisms including, besides Rhizobium, Azotobacter, Clostridium and Anabaena.

SUMMARY

1. The significance of molybdenum in the metabolism and growth of *Casuarina* and *Alnus* was investigated, both for nodulated plants dependent on the fixation of elemental nitrogen and for non-nodulated plants supplied with nitrate nitrogen.

2. In both genera molybdenum was found to be necessary for each type of plant. Nitrate plants deprived of molybdenum showed abnormal visual features in the shoot and an accumulation of unreduced nitrate in the tissues. Nodulated plants developed marked nitrogen deficiency, shown to be due to a decrease in the effectiveness of the nodule tissues in fixation of nitrogen.

3. The evidence indicates that in Alnus at least molybdenum is of greater significance to nodulated plants than to those utilising nitrate nitrogen.

4. The nodules have a high accumulative capacity for molybdenum.

5. For nodulated plants the observations are wholly consistent with the view that molybdenum fulfils some essential role in the fixation of nitrogen proceeding in these non-legume nodules, and indicate that in non-nodulated plants supplied with nitrate, molybdenum is concerned with the reduction of nitrate subsequent to absorption.

ACKNOWLEDGEMENTS

The authors are indebted to Miss K. McCormick, Messrs. G. H. Dickes and D. James for technical assistance, and to Mr. R. Cowper for photography. A grant towards the cost of the coloured illustrations included

in this paper has been received from the Carnegie Trust for the Universities of Scotland.

Received July 4, 1960

REFERENCES

1 Anderson, A. J., The role of molybdenum in plant nutrition. *In:* Inorganic Nitrogen Metabolism, ed. McElroy and Glass. 728 pp. John Hopkins Univ. Press, Baltimore. (1956).
2 Bond, G., The development and significance of the root nodules of *Casuarina*. Ann. Botany (London) N.S. **21**, 373–380 (1957).
3 Bond, G., Symbiotic nitrogen fixation by non-legumes. *In:* Nutrition of the Legumes, ed. Hallsworth. 359 pp. Butterworth, London. (1958).
4 Bond, G., Fixation of nitrogen in non-legume root-nodule plants. *In* Utilization of Nitrogen and its Compounds by Plants, ed. Porter. 385 pp. Cambridge Univ. Press, London. (1959).
5 Evans, H. J., Diphosphopyridine nucleotide-nitrate reductase from soybean nodules. Plant Physiol. **29**, 298–301 (1954).
6 Ferguson, T. P. and Bond, G., Observations on the formation and function of the root nodules of *Alnus glutinosa*. Ann. Botany (London), N.S. **17**, 175–188 (1953).
7 Hewitt, E. J., Sand and water culture methods used in the study of plant nutrition. Commonwealth Bur. Hort Plantation Crops Tech. Commun. no **22**, 241 pp. (1952).
8 Hewitt, E. J., Symptoms of molybdenum deficiency in plants. Soil Sci. **81**, 159–171 (1956).
9 Hewitt, E. J., Some aspects of mineral nutrition in legumes. *In:* Nutrition of the Legumes, ed. Hallsworth. 359 pp. Butterworth, London (1958).
10 Hewitt, E. J. and Hallas, D. G., The use of *Aspergillus niger* (Van Tiegh.) M strain as a test organism in the study of molybdenum as a plant nutrient. Plant and Soil **3**, 366–408 (1951).
11 Jacobsen, L., Maintenance of iron supply in nutrient solutions by a single addition of ferric potassium ethylenediaminetetraacetate. Plant Physiol. **26**, 411-413 (1951).
12 Jensen, H. L., Nitrogen fixation in leguminous plants, VI. Proc. Linn. Soc. New South Wales **70**, 203–210 (1946).
13 Leaf, G., Gardner, I. C., and Bond, G., Observations on the composition and metabolism of the nitrogen-fixing root nodules of *Alnus*. J. Exptl. Botany **9**, 320–331 (1958).
14 Leaf, G., Gardner, I. C., and Bond, G., Observations on the composition and metabolism of the nitrogen-fixing root nodules of *Myrica*. Biochem. J. **72**, 662–667 (1959).
15 Mulder, E. G., Bakema, K., and Van Veen, W. L., Molybdenum in symbiotic nitrogen fixation and in nitrate assimilation. Plant and Soil **10**, 319–334 (1959).
16 Piper, C. S. and Beckwith, R. S., A new method for determination of small amounts of molybdenum in plants. J. Soc. Chem. Industry **67**, 374–378 (1948).
17 Sørenson, C., The xylenol method and determination of nitrate in beets. Physiol. Plantarum **9**, 304–320 (1956).
18 Stout, P. R. and Meagher, W. R., Studies of the molybdenum nutrition of plants with radio-active molybdenum. Science **108**, 471–473 (1951).
19 Templeman, W. G., Culture of plants in sand and solutions. Jealott's Hill Research Sta. Bull. No. **2**, 28 pp. (1941).
20 Wilson, P. W., Asymbiotic nitrogen fixation. *In:* Encyclopedia of Plant Physiology **8**, ed. Ruhland. 1310 pp. Springer, Berlin. (1958).

EFFECT OF NUTRITIONAL STRESS ON PLANT COMPOSITION

II. CHANGES IN SUGAR AND AMIDE NITROGEN CONTENT OF NORMAL AND SULFUR-DEFICIENT ALFALFA DURING GROWTH

by V. V. RENDIG and E. A. McCOMB

Department of Soils and Plant Nutrition,
University of California, Davis, California

INTRODUCTION

Some effects of sulfur deficiency on the amounts of sugar and of certain nitrogen fractions present in alfalfa were reported in a previous publication [19]. Plants grown in solutions with a low level of sulfate sulfur contained relatively larger amounts of amide nitrogen but lesser amounts of sugar than similar plants grown in cultures containing an adequate sulfur supply. These effects are generally similar to those reported from cotton nutrition studies by Ergle and Eaton [6].

Further studies were undertaken to find which particular sugars were diminished by lack of sulfur and whether the manifestations of the deficiency were evident throughout the whole period of vegetative growth. It was hoped that such information would help to distinguish the primary effects of sulfur deficiency from subsequent secondary symptoms due to metabolic disturbances induced by the former.

In most plants the predominant sugars extractable by 80% alcohol are glucose, fructose, and sucrose [24]. The evidence indicates that sucrose [4] or possibly its phosphate ester [11] is the main form in which carbohydrates are translocated in higher plants. In alfalfa both reducing sugars and sucrose have been reported to be present in higher concentrations in the stem than in the leaf [9]. These workers also found that the percentages of sucrose exceeded those of the

reducing sugars in both leaves and stems. This is in contrast to the results obtained by Mason and Maskell [14] in their classic studies with the cotton plant. In this plant it was found that the amount of reducing sugars in the leaf greatly exceeded that of sucrose.

MATERIALS AND METHODS

Alfalfa (*Medicago sativa*, var. Caliverde) was grown in nutrient-culture solutions in the greenhouse in a manner similar to that previously described [19]. Each shoot was tagged on the date of its emergence. All samples were collected in the middle of the afternoon. Two separate experiments were conducted, each involving a comparison of the composition of alfalfa grown with and without an adequate supply of sulfur.

In the first experiment, which was carried out in October, 1958, the two solutions contained 250 μM and 5 μM S per liter, respectively. It was necessary to include some sulfate even in the low-sulfur cultures in order to be assured of getting enough plant material for the analyses. The plants were harvested, dried, and ground as in the previous tests [19], and the methods of analysis of the A.O.A.C.[1] for the extraction, clarification with ion-exchange resins, and Somogyi micro copper determinations of total and reducing sugars were followed. An adaptation of the method of Pucher *et al.*[18] was used to determine "amide" nitrogen. The data obtained is shown on a time *vs.* composition plot and a line of best fit was drawn visually to help discern the trends which occurred.

In the second experiment, which was carried out in September, 1959, the solutions used contained 250 μM S per liter and no added sulfate, respectively. Fresh tissue extracts were examined by use of paper-chromatographic techniques according to the following procedure:

Directly after harvest, the leaves and petioles (shoot tips not included) were separated from the stems. A liquid fraction was immediately expressed from the tissue [12] and duplicate 25-μl aliquots were transferred directly to Whatman # 1 chromatographic paper. Standards of glucose, fructose, and sucrose were included on each paper. The chromatographic papers were irrigated with pyridine, ethyl acetate, and water (8 : 2 : 1) for 16 hours [10], washed with anhydrous ether, and air dried. Reducing-sugar areas were developed by dipping in 2% aniline and trichloroacetic acid in ethyl acetate [13], air drying, and heating at 85°C for 5 minutes. Sucrose and fructose spots were detected using 0.2% resorcinol in ethyl alcohol 2N HCl solution [17] as a dip, air drying, and heating at 90°C for about 5 minutes. Ketoheptose spots were detected on the chromatograms by using an orcinol dip as recommended by Bevenue and Williams [2]. The location and color of the areas were compared with authentic samples of mannoheptulose and of sedoheptulosan anhydride which had been treated with 3N HCl at room temperature [22].

An estimation of the relative quantity of each sugar was made by visual comparison with a spot from a definite concentration of a known sugar developed on the same sheet.

For the detection of amides duplicate 10-μl aliquots of the expressed juice were spotted on Whatman # 1 paper which was irrigated with liquefied phenol:water in the ratio of 10 to 2 (Ref.[3]). The papers were sprayed with ninhydrin (0.2% in EtOH), heated at 100°C for 10 minutes and the spots compared with standards run on the same paper. Two-dimensional chromatograms were also run for the detection of amides and amino acids and these qualitatively substantiated the results obtained using the one-dimensional procedure.

Chromatographic comparisons were made of the sugar contents of freshly expressed juice, boiling alcohol extracts of tissues frozen immediately after harvest in dry-ice, juice expressed from the frozen tissues, and alcohol extracts of oven-dried tissue. The relative amounts and number of sugars found by the various sampling procedures were in good agreement. Invertase activity, as would be shown by an increase in glucose and fructose, was not observed when an aliquot of freshly expressed juice was superimposed on a sucrose standard and the chromatogram air-dried, irrigated and developed as previously described.

EXPERIMENTAL RESULTS

The total growth of the plants was as expected from the previous findings [19]. Plants grown in cultures to which 5 μM per liter of sulfate sulfur or no sulfur was added were considerably shorter and the leaves were small and chlorotic compared with those grown in cultures to which 250 μM per liter of sulfate sulfur was added. The chlorotic condition was not particularly severe initially in the plants grown in cultures to which 5 μM per liter of sulfate sulfur was added but it became more marked as the plants developed. When the last samples were collected in both experiments flower buds were just starting to appear on some of the shoots of the plants provided with adequate sulfur.

The effects of sulfur deficiency on the percent "amide" nitrogen in the stems and leaves during the course of the plants development are shown in Figures 1A and 1B, respectively. The changes which occurred in the normal and in the sulfur-deficient plants during the vegetative growth period are quite different. While in plants well supplied with adequate sulfur the percent of "amide" nitrogen decreases with age, in those provided with a low level of sulfur this trend is apparently reversed. As shown in previous work [19] "amide" nitrogen may account for as much as 30 percent of the total nitrogen in sulfur-deficient alfalfa.

The effect of sulfur deficiency on reducing-sugar content is less

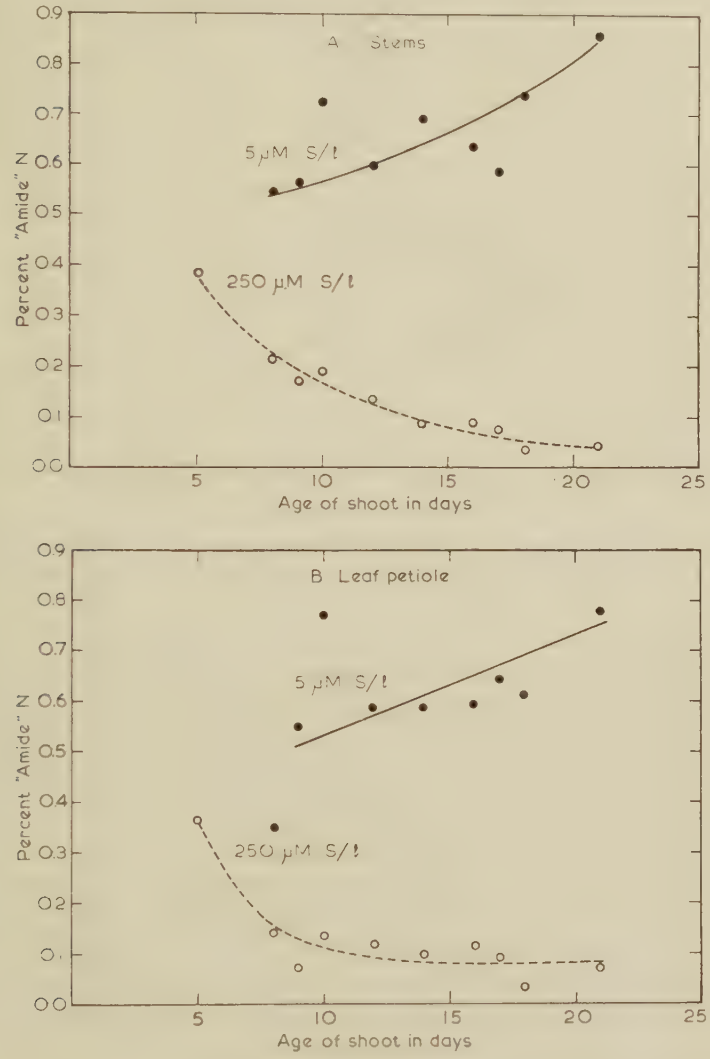


Fig. 1. Changes in the "amide" nitrogen content of the stem (A) and leaf petiole (B) fractions of alfalfa grown in nutrient solutions containing two levels of sulfate sulfur.

well defined. As reported previously [19] the amount of this kind of sugar in the mature alfalfa plant is so low that small changes are difficult to detect by available chemical methods. In the present study it was found that the reducing sugars generally accounted for less than 0.5 per cent of the dry weight of the leaves and neither

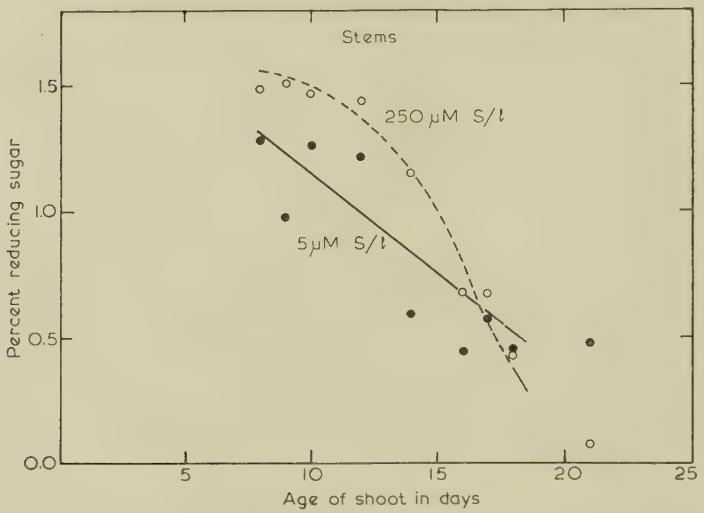


Fig. 2. Changes in the reducing sugar content of the stem fraction of alfalfa grown in nutrient solutions containing two levels of sulfate sulfur.

consistent effects of sulfur supply nor consistent changes with time were discernible. The percent of reducing sugar in the young stem was about three times that in leaves of the same age (Figure 2). During the early stages of development an inadequate sulfur supply appeared to reduce the level of reducing sugar in the stems but this effect disappeared as the shoot aged and the level in both low-sulfur and adequate-sulfur plants dropped.

The percent of non-reducing sugar in the plant are shown in Figures 3a and 3b for the stems and leaves, respectively. An inadequate supply of sulfur appears to diminish this fraction at an earlier stage in the leaves than in the stems. During the vegetative growth period this fraction increases, the rate being less pronounced if the plant does not receive adequate sulfur.

A typical growth curve was obtained when weight of leaves or stems per shoot *vs.* age of shoot was plotted. The curve showed that change in composition was less during the period of maximum rate of growth.

The results obtained using fresh plant material and chromatographic detection of glucose, fructose, and sucrose are shown in Figure 4. The size of the spot is an approximate representation of the relative amounts of sugar detected on the chromatogram. A

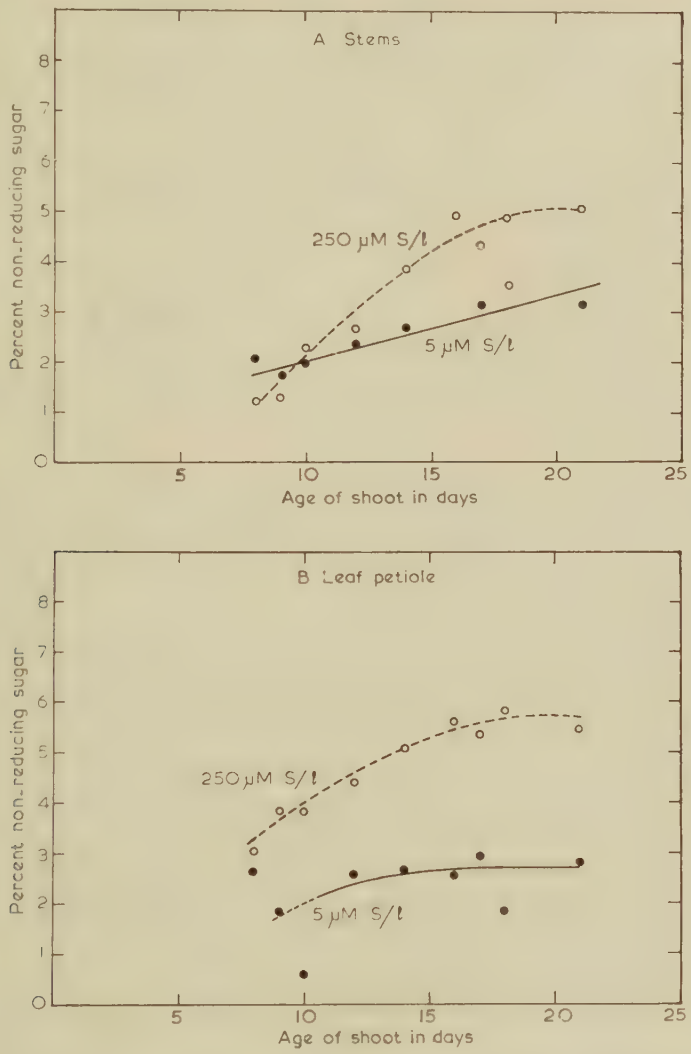


Fig. 3. Changes in the non-reducing sugar content of stem (A) and leaf petiole (B) fractions of alfalfa grown in nutrient solutions containing two levels of sulfate sulfur.

much greater effect of a lack of sulfur on glucose and fructose was revealed than had been indicated from the analysis for reducing sugar by the copper-reduction method in the first experiment. This effect was particularly evident in the young tissue and may be

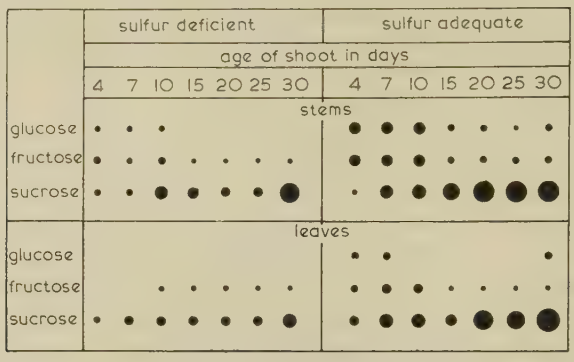


Fig. 4. The relative glucose, fructose, and sucrose contents of stems and leaves of sulfur-deficient and sulfur-adequate alfalfa with increasing age of the shoot.

explained by the more marked deficiency of sulfur in the material examined chromatographically. Samples analyzed by the copper reduction method were from plants which were grown in the presence of a small amount of sulfur.

Chromatography also indicated that the high "amide" nitrogen values found previously in plants grown in solutions receiving no sulfur were apparently largely due to the presence of asparagine.

A greenish blue spot located in a position corresponding to that of authentic mannoheptulose was observed on chromatograms of expressed juice from leaves of 1- to 2-week-old shoots. Evidence for the presence of sedoheptulose in leaf tissue was also indicated, but its occurrence was less consistent than that of mannoheptulose. Nordal and Benson [16] have isolated mannoheptulose from avocado leaves, and Williams, Potter, and Bevenue [25] have detected ketoheptoses in alfalfa leaves.

DISCUSSION

By virtue of the roles which sulfur assumes in plant metabolism, predictions can be made regarding possible effects of a shortage of this element on plant composition. Quantitatively most of the sulfur in alfalfa can be accounted for in ninhydrin-reactive substances [21] presumably as methionine, cysteine, and cystine. Since these amino acids are present largely in proteins, the synthesis of proteins or at least sulfur-containing proteins would be blocked by a lack of

sulfur. The accumulation of amide noted in the present experiments as well as in others [5 15 23] is usually attributed to such blocking. Further evidence which would be consistent with this kind of response was obtained by Mertz and Matsumoto [15]. They found in alfalfa leaf-cytoplasm preparations that the electrophoretically separated protein fraction present in greatest abundance in normal plants was present in much lesser amounts in plants grown under conditions of sulfur inadequacy. The abundance of another protein fraction was at the same time increased. Other manifestations of sulfur deficiency included an increase in arginine content and a decrease in glutamic acid. Similar effects have been reported for other plant species [5].

A deficiency severe enough to disrupt the normal course of protein synthesis would ultimately be reflected in changes in the kinds and amounts of various carbohydrates as well. This prediction would seem to be a reasonable one from a consideration of the interconnecting pathways of protein and carbohydrate metabolism which seems to be evident in a wide variety of living organisms [8]. The chlorotic condition of their leaves is visual evidence that the photosynthetic mechanism of sulfur-deficient plants is disturbed. Evidence that chlorosis might be brought about by an accumulation of toxic levels of non-protein nitrogen compounds has been reported [20] but the possibility of a direct effect of sulfur deficiency on carbohydrate metabolism cannot yet be rejected. Since many complex physiological mechanisms are involved, it would be presumptive indeed to consider the data obtained in the present study sufficiently conclusive to verify or disprove the hypothesis that the abnormal sugar levels in sulfur-deficient plants are a consequence of an abnormal nitrogen metabolism. To speculate as to whether or not the data fit the general pattern that might obtain, however, would seem pertinent.

Unlike the results found in the case of nitrogen metabolism, sulfur deficiency did not result in an accumulation to above-normal levels of any sugar determined. Generally in fact, the amounts of sugars were less in the sulfur-deficient than in the normal plant. If this were a secondary effect resulting from the loss of the ability of the plant to synthesize protein, the effects on the various sugars might be expected to be more or less similar. The glucose content, however, appeared to be more severely affected by a lack of sulfur

than did fructose or sucrose. Another factor of importance to consider is that a relatively large amount of fixed carbon in the sulfur-deficient plant was present as asparagine whose carbon chain must originate somewhere along the pathway leading from the photosynthetic reaction. Further tests are being designed in which it is hoped that the separate effects on protein and carbohydrate metabolism can be observed.

While interpretations based on comparative behavior of plants grown in independent experiments must be made with caution, it is interesting to compare the results reported here with those obtained when other nutrient deficiencies are involved. Ergle and Eaton [7] found that cotton grown in solutions low in phosphate contained four times as much reducing sugar per unit dry weight as similar plants grown in solutions containing adequate phosphate. Earlier these investigators [6] had reported that a low sulfur supply had an effect on the composition of the cotton plants similar to that on the composition of alfalfa noted in the present experiment.

SUMMARY

Nutritional stress imposed on the alfalfa plant by an inadequate supply of sulfur altered the course of changes in composition which normally accompany its ontogenetic development. Of the constituents examined the greatest alteration occurred in the "amide" nitrogen (asparagine) fraction. During the course of development of plants well supplied with sulfur the level of this nitrogen fraction decreased but in the sulfur-deficient plant it increased. Among the sugars the effect of greatest magnitude appeared to be the almost complete absence of glucose in the stems of young plants not receiving adequate sulfur. Subsequent tests have revealed that the divergence is even more dramatic if only the upper stems are used for this comparison since the reducing sugars normally are most abundant in this part of the plant. The relationship of sulfur deficiency to the occurrence of sucrose and fructose appeared to be less intimate. The presence of these sugars in sulfur-deficient plants was always indicated but at subnormal levels. Throughout the course of ontogenetic development the fluctuations in sucrose and fructose followed a pattern similar to that shown in the sulfur-adequate plants.

Received June 30, 1960.

REFERENCES

1 Association of Official Agricultural Chemists. Official and Tentative Methods of Analyses. Washington, D.C. 8th Ed. (1955).

2 Bevenue, A. and Williams, K. T., Further evidence indicating the specificity of the orcinol spray reagent for ketoheptoses on paper chromatograms. Arch. Biochem. Biophys. **34**, 225–227 (1951).

3 Block, R. J., Durrum, E. L., and Zweig, G., Paper Chromatography and Paper Electrophoresis. Second Edition. Academic Press, New York (1958).

4 Bonner, J., Plant Biochemistry. p. 30. Academic Press, New York (1950).

5 Coleman, R. G., The effects of sulfur deficiency on the free amino acids of some plants. Australian J. Biol. Sci. **10**, 50–56 (1957).

6 Ergle, D. R. and Eaton, F. M., Sulphur nutrition of cotton. Plant Physiol. **26**, 639–654 (1951).

7 Ergle, D. R. and Eaton, F. M., Aspects of phosphorus metabolism in the cotton plant. Plant Physiol. **32**, 106–113 (1957).

8 Fruton, J. S. and Simmonds, S., General Biochemistry. John Wiley and Sons, Inc., New York (1958).

9 Hirst, E. L., MacKenzie, D. J., and Wylam, Clare B., Analytical studies on the carbohydrates of grasses and clovers. IX. Changes in carbohydrate composition during the growth of lucerne. J. Sci. Food Agr. **10**, 19–26 (1959).

10 Jermyn, M. A. and Isherwood, F. A., Improved separation of sugars on paper partition chromatogram. Biochem. J. **44**, 402–407 (1949).

11 Kursanov, A. L., Recent advances in plant physiology in the U.S.S.R. Ann. Rev. Plant Physiol. **7**, 401–436 (1956).

12 McComb, E. A. and Rendig, V. V., Techniques for extracting juice from plant tissue. Chemist Analyst (in press) (1960).

13 McCready, R. M. and McComb, E. A., Quantitative determination of sugars on paper chromatograms by a reflectance method. Anal. Chem. **26**, 1645–1647 (1954).

14 Mason, T. G. and Maskell, E. J., Studies on the transport of carbohydrates in the cotton plant. I. A study of diurnal variation in the carbohydrates of leaf, bark and wood, and of the effects of ringing. Ann. Botany **42**, 189–253 (1928).

15 Mertz, E. T. and Matsumoto, H., Further studies on the amino acids and proteins of sulfur-deficient alfalfa. Arch. Biochem. Biophys. **63**, 50–63 (1956).

16 Nordal, A. and Benson, A. A., Isolation of mannoheptulose and identification of its phosphate in avocado leaves. J. Am. Chem. Soc. **76**, 5054–5055 (1954).

17 Partridge, S. M., Filter paper partition chromatography of sugars. I. General description and application to the qualitative analysis of sugars in apple juice, egg white, and foetal blood of sheep. Biochem. J. **42**, 238–248 (1948).

18 Pucher, G. W., Vickery, H. B., and Leavenworth, O. S., Determination of ammonia and amide nitrogen in plant tissue. Ind. Eng. Chem. Anal. Ed. **7**, 152–156 (1935).

19 Rendig, V. V. and McComb, E. A., Effect of nutritional stress on plant composition I. The interaction of added nitrogen with varying sulfur supply. Soil Sci. Soc. Am. Proc. **23**, 377–380 (1959).

20 Steinberg, R. A., Bowling, J. D., and McMurtrey, J. E., Jr., Accumulation of free amino acids as a chemical basis for morphological symptoms in tobacco manifesting frenching and mineral deficiency symptoms. Plant Physiol. **25**, 279–288 (1950).

21 Steward, F. C., Thompson, J. F., Miller, F. K., Thomas, M. D., and Hendricks, R. H., The amino acids of alfalfa as revealed by paper chromatography with special reference to compounds labelled with S 35. Plant Physiol. **26**, 123–135 (1951).

22 Stewart, L. C., Richtmyer, N. K., and Hudson, C. S., The oxidation of volemitol

by Acetobacter suboxydans and by A. xylinium. J. Am. Chem. Soc. **71**, 3532, footnote 21 (1949).

23 Thomas, M. D., Hendricks, R. H., and Hill, G. R., Sulfur metabolism in alfalfa. Soil Sci. **70**, 19–26 (1950).

24 Williams, Kenneth T. and Bevenue, Arthur, Use of ion exchange resins for the removal of non-sugar reducing substances in the analysis of fresh leafy plant materials for sugars. J. Assoc. Official Agr. Chemists **34**, 817–821 (1951).

25 Williams, Kenneth T., Potter, Earl F., and Bevenue, Arthur, A study, by paper chromatography, of the occurrence of non-fermentable sugars in plant materials. J. Assoc. Official Agr. Chemists. **35**, 483–486 (1952).

Plant and Soil XIV, no. 2 April 1961

THE EFFECT OF COLCHICINE AND INDOLYL ACETIC ACID ON DIPLOID AND TETRAPLOID STRAINS OF RED AND WHITE CLOVERS IN ASEPTIC AND POT CULTURE

by J. B. WEIR

Botany Department, University College of Rhodesia and Nyasaland, Salisbury, Southern Rhodesia

INTRODUCTION

Colchicine is a mitotic poison, known to induce doubling of the chromosome number in many types of plant cells. It was shown by Wipf and Cooper [23 24] that cells with the tetraploid number of chromosomes were occasionally present at the level of root-hair production in different diploid legumes. Occasionally also triploid cells were found in the meristem (Bond [2]). These disomatic cells appeared to be centres of nodule production, with the infected host cells of the nodule also showing the disomatic chromosome number. This was also true for polyploid plants (Wipf [22], Funcke [10]) where the infected cells of a tetraploid plant showed the octaploid chromosome number. This led to investigations on the effect of colchicine on nodule production.

It was shown by Bonnier [4] that in aseptic culture, a concentration of one part in twenty-five thousand colchicine stimulated the earlier formation of a larger number of nodules on *Trifolium pratense* than was found on untreated controls but that at a concentration of one part in five thousand abnormalities of the aerial parts of the plant developed. Migahid *et al.*[15] stated that if Trifolium seeds were soaked for 24 hours in a 0.5% solution of colchicine, there were five times as many nodules on treated plants as on untreated ones after 3 weeks, and three times as many after 4 weeks, *i.e.* again, that earlier formation of a larger number of nodules was

induced. In the present experiment, the effect of different concentrations of colchicine on diploid and tetraploid varieties of red and white clovers respectively was studied in both non-sterile pot culture and in aseptic culture.

Also it has been shown that infection occurs via the root hairs, which are usually, but not invariably, first of all deformed due to a secretion of indolyl acetic acid (IAA) by the bacteria (Kostermans [13], Thimann [21], Georgi and Beguin [11]). The present experiment also investigated the effect of different concentrations of IAA on diploid and tetraploid red and white clover plants in both non-sterile pot culture and in aseptic culture.

MATERIALS AND METHODS

(*A*). A tetraploid strain of red clover (*Trifolium pratense*), kindly supplied by Dr. A. Evans of the Welsh Plant Breeding Station, Aberystwyth, and a diploid variety of *Trifolium pratense* var. Chilean Red ($2n = 14$) were used as the test plants. The seeds were soaked overnight and placed in pots each containing 400 g of unsterilised soil, which were then placed in the bottom half of a petri plate, to which water was added when necessary. After a fortnight the diploids were at the first-leaf stage, while the tetraploids were much slower to germinate and still had only cotyledons. At this stage a two-day-old inoculum of an effective strain of *Rhizobium trifolii* in 5 ml of yeast-mannitol water was added to each pot. A week later *i.e.* at three weeks after planting, the following treatments were carried out: —

a) 10 ml of 1/10,000 colchicine was added to 6 pots containing *diploid* plants
b) 10 ml of 1/10,000 IAA ,, ,, ,, ,, ,, ,, ,,
c) 10 ml of 1/5,000 IAA ,, ,, ,, ,, ,, ,, ,,
d) (control) 10 ml of tapwater ,, ,, ,, ,, ,, ,, ,,

The same treatments were applied to pots with *tetraploid* plants.

The seedlings were later thinned out to 3 per pot, giving eighteen plants per treatment.

These treatments were repeated at weekly intervals for 16 weeks after which the plants were harvested, roots carefully washed, nodules counted, and distribution noted. Total plant dry weights were also estimated.

On the basis of the results of this experiment, a further experiment was carried out, using a tetraploid strain of white clover (*Trifolium repens*) from the same source and a diploid strain S 100. In this case, the plants were treated with a) 1/10,000 colchicine, b) 1/5,000 colchicine, c) 1/10,000 IAA, d) 1/5,000 IAA, e) tapwater (as a control).

Again 10 ml of each was added at weekly intervals, and at the 16 week stage the plants were harvested.

(*B*). Using an aseptic technique (Weir[25]), diploid and tetraploid white clover plants were grown in a N-free mineral-agar medium, inoculated with

an effective strain of the appropriate bacterium and given the same treatments as above, that is 1/10,000 colchicine, 1/5,000 colchicine, 1/10,000 IAA and 1/5,000 IAA respectively were introduced into the medium. This was run with a control in which 10 ml of tapwater was added to the medium. After a fortnight, however, it became apparent that the higher concentration of both IAA and, to a lesser degree, colchicine, was too high for growth of the plants in aseptic medium, as the roots were stunted and malformed and the plants dying off. This experiment was therefore repeated, using the concentrations 1 ppm colchicine, 1/10,000 colchicine, 1 ppm IAA, 1/10,000 IAA, and a control. Again there were 6 jars per treatment and 3 plants per jar. These were harvested at an early stage of development (8 weeks) and the nodule numbers counted.

RESULTS

In the control plants of the red clovers, the diploids had an average nodule number of 37.0 and the tetraploids an average of 53.3 (Table 1). The addition of 1/10,000 colchicine caused a distinct increase in nodulation in both cases, (by 148 per cent in the case of the diploids and by 254 per cent in the case of the tetraploids). 1/10,000 IAA also caused a slight increase in nodulation in both cases, but the stronger concentration of IAA caused a slight decrease, though with the numbers of plants tested, this difference was not statistically significant.

TABLE 1

Average nodule numbers on diploid and tetraploid red clover plants grown in pot culture for 16 weeks

Treatment	No. of chromosomes	
	2n	*4n*
Control	37.0	53.3
1/10,000 colchicine	92.1	188.5
1/10,000 IAA	45.6	93.0
1/5,000 IAA	35.5	49.7

In the white clovers (Table 2), diploid control plants had an average nodule number of 123,6 while tetraploids were considerably lower, with an average of 52.4 nodules per plant. 1/10,000 colchicine increased the nodule numbers considerably in both cases, as with the red clovers, while the additional treatment of a stronger concentration of colchicine showed an even greater enhancement of nodule numbers.

As with the red clovers, 1/10,000 IAA caused a slight increase in nodule numbers. At a concentration of 1/5,000 IAA with the diploids there was a slight depression of nodule numbers below those of the controls, but with the polyploids there was a very slight increase. However, as was stated, before, this cannot be said to be statistically significant in either case.

TABLE 2

Average nodule numbers on diploid and tetraploid white clover plants grown in pot culture for 16 weeks

Treatment	No. of chromosomes	
	2*n*	4*n*
Control	123.6	52.4
1/10,000 colchicine	208.4	99.2
1/5,000 colchicine	292.9	118.2
1/10,000 IAA	165.9	68.4
1/5,000 IAA	102.1	58.7

A study of the dry weights of the red clover plants showed that the diploid control plants had an average weight of 0.183 g per plant (Table 3), while the tetraploids were rather higher at 2.28 g. The addition of 1/10,000 colchicine which had caused an increase in the nodule numbers in both cases, also caused a considerable increase in the dry weights, as did the addition of 1/10,000 IAA, to an even greater degree, although the increase in nodulation had not been so great. Although 1/5,000 IAA had resulted in a slight drop in nodule numbers below those of the controls, there was nevertheless a slight increase in the dry weights in both the diploids and the tetraploids, therefore it appears that the dry weights are not entirely dependent on the nodule numbers.

TABLE 3

Average dry weight in g per plant of red clover plants grown in pot culture for 16 weeks

Treatment	No. of chromosomes	
	2*n*	4*n*
Control	0.183	0.28
1/10,000 colchicine	0.271	0.495
1/10,000 IAA	0.28	0.61
1/5,000 IAA	0.201	0.32

In the diploid white clover plants, the average dry weight per plant was 0.504 g (Table 4), while the tetraploids were unexpectedly lower, at 0.403 g. In both cases the addition of 1/10,000 colchicine increased the dry weights, to 1.14 g and 0.79 g respectively, while the addition of the stronger concentration of colchicine, which had caused an even greater enhancement of nodule numbers, gave an increase in dry weight over that of the controls in the diploids, but was slightly less than those plants treated with 1/10,000 colchicine. (Again the differences were not statistically significant). With the tetraploids, the greater concentration of colchicine gave a greater average dry weight. 1/10,000 IAA caused an increase in the dry weights of both the diploids and tetraploids over the controls, while the higher concentration of IAA resulted in a slight decrease below that of the controls in the diploids and a slight increase in the case of the tetraploids.

TABLE 4

Average dry weight in g per plant of white clover plants grown in pot culture for 16 weeks

Treatment	No. of chromosomes	
	2n	*4n*
Control	0.504	0.403
1/10,000 colchicine	1.14	0.79
1/5,000 colchicine	0.96	0.954
1/10,000 IAA	0.732	0.665
1/5,000 IAA	0.334	0.560

In every case the nodules were of the effective type, with pink or green colouration, though the nodules on the tetraploid plants tended often to be rather larger than those on the diploids.

In the case of the plants grown in aseptic culture for 8 weeks, the average number of nodules per diploid control plant was 3.2, while that of the tetraploids was 2.35 (Table 5). A concentration of one in a million colchicine caused earlier nodule initiation, a point which could not be studied in pot culture, and in both the diploids and the tetraploids there was an increase in nodule numbers over the controls. 1/10,000 colchicine again caused a greater average nodule number than the controls, but this was not so marked. In contrast to the pot experiments, in aseptic culture even a low concentration (1 ppm) of IAA almost completely inhibited nodule formation, and

also root development, while at the higher concentration 1/10,000 this was even more marked.

TABLE 5

Average nodule numbers on diploid and tetraploid white clover plants grown in aseptic culture for 8 weeks

Treatment	No. of chromosomes	
	2*n*	4*n*
Control	3.2	2.35
1 ppm colchicine	6.3	3.33
1/10,000 „	4.0	2.88
1 ppm IAA	0.5	0.2
1/10,000 IAA	0.0	0.3

DISCUSSION

A) The effect of colchicine on growth and nodulation of red and white clovers

The fact that both nodule numbers and dry weights in the white clovers were higher than those of the red clovers may be put down to the time of year at which the experiments were carried out. The experiment with the red clovers ran through August till November, which is the "dead" season in the Federation, while that with the white clovers ran from November until February, which is the peak growing season.

In the non-sterile pot experiment with the white clover plants, fewer nodules were formed on the tetraploid plants than on the diploids, but these tended to be larger. This is in accord with previous work (Nillson and Rydin [16]; Nutman [18]; Weir [26]). In aseptic culture, the tetraploid white clovers again showed slightly fewer nodules than the diploids. However, the red clover plants showed a converse effect, with greater nodule numbers on the tetraploid plants than on the diploids. It is difficult to explain this, but it seems possible that as rhizobial strains do not always respond in the same way to tetraploid plants as they do to diploids (Weir [26]), the bacterial strain used in this case was initially more infective with the tetraploid plants than with the diploids.

In both non-sterile and aseptic culture, a low concentration of colchicine markedly increased nodule numbers on diploid and

tetraploid plants, but optimum concentrations varied in the two different types of culture. In aseptic culture a concentration of one in five thousand colchicine had an adverse effect on plant growth. This agrees with the findings of Bonnier [4].

In pot culture however, this was not found even with repeated weekly applications of 10 ml of one in five thousand colchicine. This may be due to a more rapid breakdown of the colchicine in the soil by micro-organisms, than in aseptic culture.

The abnormal cytology of nodules was first commented on by Fred in 1908, while more recent studies have added a certain amount to our knowledge. It was found (Wipf [22], Wipf and Cooper [23 24]) that for a wide range of legumes, inoculated with effective or ineffective strains of bacteria, the host cells in the central tissue of the nodule contain double the number of chromosomes present in the rest of the root. These relationships were found for polyploid as well as diploid hosts, so that a tetraploid host has octaploid cells in the central infected region of the nodule (Funcke [10]). These disomatic cells arise from single disomatic cells which are already present in the root cortex before infection, and indeed, in the uninoculated root. They are commonest in the root-hair zone, and are often associated with lateral roots. Occasional triploid cells have even been found in the meristem (Bond [2]). These disomatic cells are stimulated to divide by the approach of an infection thread. Those threads penetrating the cortex away from the disomatic cells cause no stimulation, and do not give rise to nodules. Presumably therefore, any increase in the numbers of these disomatic cells, such as might be caused by applications of colchicine, will lead to an increase in nodule numbers. Further cytological investigations of this point are necessary however.

B) The effect of indolyl acetic acid on growth and nodulation of red and white clovers

Thimann's [21] experiments indicated that the initiation of the lateral primordium giving rise to the nodule was induced from outside the root by the secretion of IAA by the nodule bacteria. Chen [7] confirmed that nodule bacteria produced a growth substance, and stated that ineffective bacterial strains which did not lead to N-fixation, nevertheless were equally efficient at producing growth stimulants. This was also found by Georgi and Beguin [11].

Link [14] extracted IAA from cultures of *Rhizobium phaseoli*, and tested this by application to Avena coleoptiles. Other bacteria have also been found to produce growth substances, *e.g. Aerobacter aerogenes* and *Escherichia coli* (Burkholder [5]), and *Bacillus radiobacter* (Georgi and Beguin [11]). However, these growth substances are produced in extremely minute quantities by bacteria and at higher concentrations many of these are known to have inhibitory effects on root growth. Bonner and Koepfli [3] showed that several such substances, including IAA, were active in root inhibition. It has also been shown that, in the field, many of the growth substances which are used as herbicides such as substituted phenoxyacetic and phenoxybutyric acids are toxic to white clover seedlings (Holly [12], Ochiltree [19], Scragg [20], Fletcher *et al.*[9]). However, Fletcher *et al.*[8] also found that in aseptic culture, when the herbicides were incorporated in the rooting medium, the relative toxicities of various substances were not the same as was found in soil culture. These workers considered that the "aseptic" findings represented a true measure of the toxicity of the substance, whereas in the field, the toxicity would depend largely upon the relative persistence in the soil. This appears to be the case in the present experiment, both with regard to colchicine and IAA, but more especially the latter, where in aseptic culture, concentrations as low as 1 ppm inhibited root growth and nodulation, while in soil cultures higher concentrations caused even a slight stimulatory effect. This discrepancy between results in soil culture and those in aseptic culture has been found by other workers (Weir [25]). It is known that many growth substances undergo a gradual inactivation in soil (Nutman *et al.*[17], Audus [1]), and that this breakdown is largely biological in character. Obviously, in aseptic culture, such a biological breakdown will not occur so rapidly as only the nodule forming organism is present. Also Bonner and Koepfli [3] stated that IAA was more effective in inhibiting root growth in an acid medium. In aseptic culture, the pH of the medium tends to fall, and often the pCO_2 inside the container may be greater than that of the outside atmosphere, thus making conditions favourable for inhibition of root growth. There is fairly general agreement with regard to the effect of growth substances on the nodulation of legumes, that where there is a reduction in nodulation, it is largely due to a reduction in root growth (Carlyle and Thorpe [6], Fletcher *et al.*[8]). It was found in the present

experiment that the higher concentrations of IAA *i.e.* 1/10,000, almost completely inhibited root growth, while even at concentrations of 1 ppm, root growth was very poor.

SUMMARY

The effect of different concentrations of colchicine and indolyl acetic acid (IAA) on diploid and tetraploid strains of red clover (*Trifolium pratense*) and white clover (*Trifolium repens*) was tested in both non-sterile pot culture and in aseptic culture. It was found that in pot culture, concentrations of one in ten thousand and one in five thousand colchicine considerably increased nodulation of both the diploid and the tetraploid plants. This was equally true of red and white clovers. In aseptic culture a concentration of one in five thousand had adverse effects on growth of white clover plants though concentrations of one ppm colchicine or one in ten thousand colchicine again produced higher nodule numbers than untreated, control plants. This was regarded as probably due to a slower breakdown of the colchicine in aseptic culture than in the soil.

In aseptic culture, even concentrations as low as one ppm indolyl acetic acid in the medium almost completely inhibited nodule formation and growth of white clover plants, but in non sterile pot culture a concentration of one in ten thousand IAA caused a slight increase in nodule number over those of controls, though a concentration of one in five thousand caused a slight depression.

Possible interpretations of these results are discussed.

Received June 21, 1960.

REFERENCES

1 Audus, L. J., The biological detoxication of 2,4-dichloro phenoxy acetic acid in soil. Plant and Soil **2**, 31–6 (1949).
2 Bond, L., Origin and developmental morphology of root nodules of *Pisum sativum*. Botan. Gaz. **109**, 411–34 (1948).
3 Bonner, J. and Koepfli, J. B., Inhibition of root growth by auxins. Am. J. Botany **26**, 557–66 (1939).
4 Bonnier, C., Action de la colchicine sur la symbiose Rhizobium – Medicago sativa et Rhizobium – Trifolium pratense. Bull. Inst. Agron. et Stas. Recherches Gembloux **22**, 167–78 (1954).
5 Burkholder, P. R., Production of growth substances by bacteria in media containing organic and inorganic N. compounds. Am. J. Botany **26**, 422–27 (1939).
6 Carlyle, R. E., and Thorpe, J. D., Some effects of ammonium and sodium 2,4-dichlorophenoxyacetates on legumes and the *Rhizobium* bacteria. J. Am. Soc. Agron. **39**, 929-36 (1947).
7 Chen, H. K., Production of growth substance by clover nodule bacteria. Nature **142**, 753-4 (1938).
8 Fletcher, W. W., Dickenson, P. B. and Raymond, J. C., The effect of certain

hormone herbicides on the growth and nodulation of *Trifolium repens sylvestre* in aseptic culture. Phyton (Buenos Aires) **7**, 121–30 (1956).

9 Fletcher, W. W., Dickenson, P. B., Forrest, J. D., and Raymond, J. C., The effect of soil applications of certain substituted phenoxyacetic and phenoxybutyric acids on the growth and nodulation of *Trifolium repens sylvestre*. Phyton (Buenos Aires) **9**, 41–46 (1957).

10 Funcke, C., Über die Chromosomenzahlen der Wurzelknöllchen experimentell erzeugter polyploider Leguminosen. Naturwissenschaften **44**, 498 (1957).

11 Georgi, C. E. and Beguin, A. E., Heteroauxin production by efficient and inefficient strains of rhizobia. Nature **143**, 25 (1939).

12 Holly, K., The effect of growth regulator herbicides on seedling clover and grasses. Proc. Brit. Weed Control Conf. 1st Conf. Margate **1952**, 116–24 (1954).

13 Kostermans, D., Over hetero-auxine. Thesis Univ. of Utrecht (1935).

14 Link, G. K. K., Role of heteroauxones in legume nodule formation, and the beneficial host effects of nodules and soil fertility. Nature **140**, 507 (1937).

15 Mirzabaf A. M., El Nadi, A. F. and Abo El Kassem, A. A., The effect of gamma radiation on bacterial nodule formation. Plant and Soil **9**, 139–144 (1958).

16 Nillson, P. E. and Rydin, C., Studies on symbiotic nitrogen fixation by a new strain of polyploid red clover. Arch. Mikrobiol. **20**, 396–403 (1954).

17 Nutman, P. S., Thornton, H. G. and Quastel, J. H., Plant growth substances as selective weed killers. Inhibition of plant growth by 2,4-dichlorophenoxyacetic acid and other plant growth substances. Nature **155**, 498–500 (1945).

18 Nutman, P. S., The influence of the legume in root nodule symbiosis. A comparative study of host determinants and functions. Biol. Rev. **31**, 109–151 (1956).

19 Oehlkers, W., The effect of growth regulator herbicides on clover. Proc. Brit. Weed Control Conf. 1st Conf. Margate **1952**, 125–128 (1954).

20 Scragg, E. B., Undersown cereals – clover and grass seedlings. Proc. Brit. Weed Control Conf. 1st Conf. Margate **1952**, 107–116 (1954).

21 Thimann, K. V., The physiology of nodule formation. Trans. Third Comm. Int. Soil Sci. New Brunswick N.S. 1939 **A**, 24–28 (1939).

22 Wipf, L., Cytological basis of [illegible] and host type in certain Leguminosae. Botan. Gaz. **101**, 51–67 (1939).

23 Wipf, L. and Cooper, D. C., Chromosome numbers in nodules and roots of red clover, common vetch and garden pea. Proc. Natl. Acad. Sci. U.S. **24**, 37–41 (1938).

24 Wipf, L. and Cooper, D. C., Somatic [illegible] in certain Leguminosae. Am. J. Botany **27**, 821–4 (1940).

25 Wipf, J. S., Investigations on the [illegible] of *Medicago sativa* L. Phyton (Buenos Aires) **10**, 153–163 (1958).

26 Wipf, J. S., A comparison of [illegible] and ineffective clover [illegible] with different mineral forms. Plant and Soil **14**, 55–67 (1961).

A

B

Plate 1

Plant and Soil XIV, no 2 April 1961

LETTER TO THE EDITORS

Some Striking Observations on the Occurrence of Fungi in Steamed Glasshouse Soil

Glasshouse soil is steamed in order to prevent a possible outbreak of soil-borne diseases of tomato. Freshly steamed soil represents a good environment for the development of micro-organisms. Even fungi which ordinarily are less able to compete sucessfully may here find a possibility for growth. For a shorter period they may dominate completely, but only until the life in the soil is beginning to attain its normal state. They will then again lose ground and finally become as unsuccessful competitors in their struggle for life as before the steaming.

These fungi do not attack the plants directly, yet they may cause considerable damage by covering large areas of the soil, thereby suffocating young seedlings and cuttings. If necessary they may be controlled by fungicides, such as *e.g.* Captan.

Under the above-mentioned conditions the following fungi were found to occur: – *Oedocephalum* sp., *Hyphelia terrestris* Fr., and *Pyronema*. Frequently there has been observed a yellowish brown fungus, which by Ivar Jørstad has been shown to be the conidial stage of *Plicaria fulva*, first described by Schneider [2] in 1954, and some years later by Rieth [1].

The development of *Plicaria fulva* is accompanied by some striking phenomena. At one time the soil may be covered with brown conidiophores, at another time – 10 to 15 hours later – the soil has grown white as if powdered with confectionary sugar. This white colour is due to the fresh development of a white mycelium and of conidiophores which are white at the early stages. After several hours the appearance of the fungus is brown again, and the development described repeats itself.

Plate 1 shows some subsequent scenes of the occurrence of *Plicaria fulva* in a tomato glasshouse. The soil consisted of 75 per cent sphagnum and 25

Plate 1. A. *Plicaria fulva* on the soil in the tomato house at 8 a.m. Where the soil is moist from watering it is covered with white mycelium and white conidiophores of this fungus. **B.** *Plicaria fulva* at the same place and on the same day at 2 p.m. The conidiophores have turned brown. (Photos by the author).

per cent field loam. A heavy application with cow manure was given, immediately prior to steaming.

The pictures were taken in May, a month after steaming and two weeks after the tomato plants had been set out.

JENS ROLL-HANSEN
State Experiment Station Kvithamar,
Stjördal, Norway.

Received July 15, 1960

Literature

1 Rieth, A., Vorkommen von *Plicaria fulva* Schneider. Die Kulturpflanze **5**, 186–189 (1957).

2 Schneider, R., *Plicaria fulva* n.sp., ein bisher nicht bekannter Gewächshausbewohner. Zentr. Bakteriol. Parasitenk. Abt. II **108**, 147–153 (1954).